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Genomic Imprinting in the Endosperm of *Arabidopsis thaliana*

Submitted by
Sally Adams BSc (Hons)
for the degree of Doctor of Philosophy
of the University of Bath
2002

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To Mum and Dad

Abstract

Some genes in mammals and plants are subject to genomic imprinting, a process by which differential epigenetic marks are imposed on male and female gametes so that one set of alleles is silenced on maternally inherited chromosomes while another is silenced on the paternally inherited chromosomes. An imbalance of parental genomes in the offspring results in abnormal development. For example, in the model plant *Arabidopsis thaliana*, seed that inherited extra maternal genomes had a low seed weight and an under proliferated endosperm, while the addition of extra paternal genomes results in larger seed with an over proliferated endosperm. Little was known about the mechanisms of genomic imprinting in plants, however DNA methylation was known to play an important role in mammalian imprinting. To investigate if it could have a similar role in plants, seed development was studied in crosses between transgenic *A.thaliana* plants (*MET1a/s* plants) with reduced methylation and wild type diploid plants. The seed produced had similar phenotypes to crosses between plants with normal methylation but different ploidies. This is consistent with a model in which hypomethylation of one parental genome prevents the silencing of alleles that would normally be active only when inherited from the other parent-phenocopying the effect of adding extra parental genomes. These results suggested an important global role for DNA methylation in the parent-of-origin effects, and by inference imprinting, in plants. We showed that *MET1* is expressed in male and female gametes, and in the early seed, but that expression becomes restricted to the embryo after late globular stage. As genomic imprinting in plants is primarily thought to target endosperm development this suggests that other factors maintain the parent-of-origin effect in later seed development. To study the role of other DNA methyltransferases a screen was conducted to identify mutants in each putative enzyme, with the aim to use these in similar experiments to those described above. We also designed and conducted a screen to identify sex-specific components of genomic imprinting system, based on the reciprocal effect on seed size in interploidy crosses. The identification and characterization of the paternal excess candidate line 22.16 highlighted the excellent potential of this screen for isolating components of the genomic imprinting system.

Abbreviations

BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CaMV	cauliflower mosaic virus
CE	chalazal endosperm
CPT	chalazal proliferating tissue
DAP	days after pollination
DIG-UTP	dioxigen-dUTP
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetraacetetic acid
EtOH	ethanol
EMS	ethylmethanosulphate
GOF	gain-of-function
h	hours
HAP	hours after pollination
HCl	hydrochloric acid
$K_3Fe(CN)_6$	potassium ferricyanide
$K_4Fe(CN)_6.3H20$	potassium ferrocyanide
LOF	loss-of-function
$MgCl_2$	magnesium chloride
ME	micropylar endosperm
min	minutes
N_2	nitrogen
NaCl	sodium chloride
NCD	nuclear cytoplasmic domain
PE	peripheral endosperm
RNA	ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
2xCol	diploid Columbia plants
4xCol	tetraploid Columbia plants
2x	diploid plants (of the ecotype C24 unless otherwise stated)
4x	tetraploid plants (of the ecotype C24 unless otherwise stated)
6x	hexaploid plants (of the ecotype Col unless otherwise stated)
[A x B]	parental plants in a cross. A is the maternal parent and B is the paternal parent

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Chapter 1

Introduction

1.1 The importance of research into seed development

Plant agriculture not only provides us with a large proportion of our nutrient intake, it is also an essential part of the world economy. In 2001, 19 million tonnes of cereal crops, at a market value of £2019 million, were produced in the United Kingdom alone (DEFRA, 2002). Thus the genes involved in seed development and their potential manipulation to improve both crop quality and yield are of extreme agricultural interest. However, research in crop species can be hindered due to the large and complex nature of their genomes. The model plant *Arabidopsis thaliana* provides an alternative and simpler system to examine such processes. The purpose of the work described in this Thesis was to use *A.thaliana* to study the process of genomic imprinting in the developing endosperm.

1.2 Seed development in angiosperms

1.2.1 Double fertilization

Seed development in angiosperms (flowering plants) is initiated by the unique process of double fertilization (reviewed in Chaudhury et al., 2001). In a cross where both parents are diploid, the pollen tube delivers two sperm cells, formed from the division of the generative cell, to the embryo sac (Figure 1.1). One sperm cell fuses with the egg cell, giving rise to the embryo. The second sperm cell fuses with the central cell, which contains the 2 polar nuclei. The eventual fusion of the 2 polar nuclei and the sperm forms the triploid primary endosperm nucleus. In most species both the central cell and egg are genetically identical as they originate from a single meiotic product. Hence the embryo and endosperm inherit the same parental genes and only differ genetically by the second maternally derived genome in the endosperm.

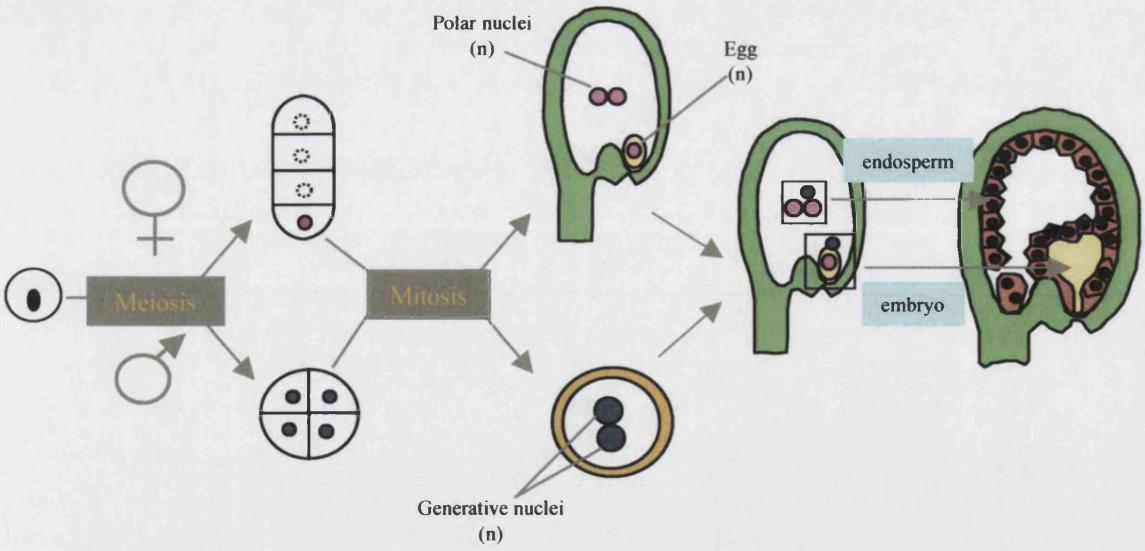


Figure 1.1

Double fertilization in *A.thaliana*. Meiosis in the female germline gives rise to an ovule containing 8 genetically identical haploid nuclei. In the male germline pollen is formed containing 3 identical haploid nuclei: 2 generative nuclei and 1 vegetative nucleus. Fertilization of the egg by the sperm gives rise to a diploid embryo. Fertilization of the 2 polar nuclei in the central cell by the second sperm results in the formation of the triploid primary endosperm nucleus. Replication and division of this nucleus leads to the formation of the endosperm.

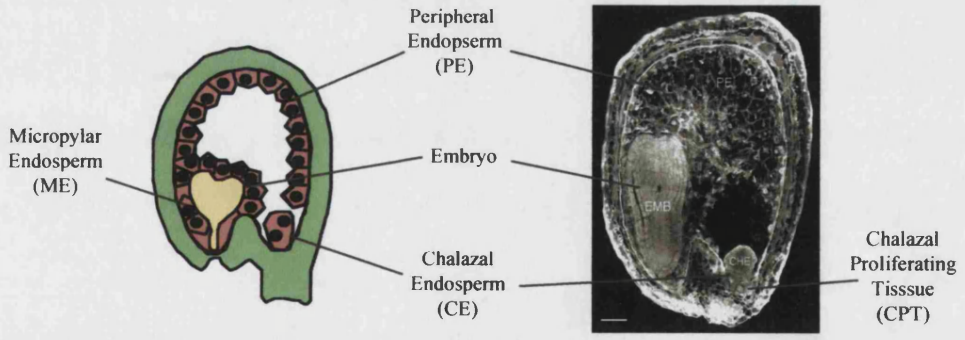


Figure 1.2

The 3 types of endosperm in *A.thaliana* seed. The micropylar endosperm (ME) surrounds the developing embryo. The peripheral endosperm (PE) constitutes the central endosperm. The final type of endosperm, the dense chalazal endosperm (CE) develops at the chalazal pole and shows close association with the maternal chalazal proliferating tissue (CPT). Bar, 50μm.

1.2.2 The function of the endosperm

Currently endosperm research is focused on plants within the two main divisions of the angiosperms – the monocotyledonous species (monocots), dominated by the economically significant cereals (such as maize, rice and barley); and the dicotyledonous species (dicots) such as the model plant *A.thaliana* (reviewed in Vinkenoog et al., 2002, Chaudhury et al., 2001). These encompass the two distinct types of endosperm development in angiosperms. Cereals and many other monocots have a persistent endosperm, which is maintained in the mature seed. In contrast, many dicots have a transient endosperm and in the mature seed of these plants the endosperm, if present at all, is reduced to a few cell layers. In the mature seed of *A.thaliana*, for example the endosperm persists as a single cell layer called the aleurone.

The endosperm is likely to play a pivotal role in resource transfer and storage in both its transient and persistent state. In cereals the endosperm stores both nutrients and hormones that are utilised by the germinating seed and provides mechanical support during early embryo growth (Lopes and Larkin, 1993; Olsen, 1998). A transient endosperm is strongly suspected to play a similar role, acting as sink for nutrients to be consumed by the developing embryo. Increasing evidence supports this hypothesis, with experiments in *A.thaliana* suggesting that the amino acid nutrition of the embryo is dependent on the endosperm (Himer et al., 1998).

Other experiments indicate that interactions between the endosperm and embryo could be important for the regulation of seed development. For example, in maize a family of novel genes have been described which are specifically expressed in the endosperm around the embryo, indicating they may be involved in interactions between the embryo and endosperm (Opsahl-Ferstad et al., 1997). Furthermore, the carrot gene EP3 (van Hengel et al., 1998), which encodes a type IV endochitinase, is strongly expressed in the seed integument and then the endosperm during seed development. Evidence points to the EP3 protein acting on arabinogalactan proteins to release oligosaccharides important for embryogenesis. Thus the endosperm is an important structure, playing a critical nutritive, structural and regulatory role during seed development.

1.2.3 The development of the endosperm

Endosperm development is characterized by four distinct phases: free nuclear, cellularization, differentiation and death, which are conserved between monocots and dicots (Berger, 1999). The duration of the phases vary from species to species (Vijayaraghavan and Prabhakar, 1984) and therefore for the purpose of clarity the description of endosperm development will concentrate on the plant under study, *A.thaliana.*

After fertilization the triploid primary endosperm nucleus replicates, without cytokinesis, to form a syncytium of many individual nuclei (Mansfield and Briarty, 1993). Early in development, despite the absence of cell walls, the endosperm develops into three morphologically distinct types. It has been suggested that the differentiation may originate from a partitioning of mRNA within the syncytium (Doan et al., 1996; Berger, 1999).

The 3 types of endosperm can be seen in the 3 regions of the developing seed (Figure 1.2). At the micropylar pole the micropylar endosperm (ME) surrounds the embryo and supporting suspensor. The central peripheral endosperm (PE) spreads as a thin layer to surround the central vacuole of the central cell. The nuclei of the PE are evenly spaced with each being encased by a sphere of cytoplasm to form a nuclear cytoplasmic domain (NCD) (Olsen et al., 1995; Brown et al., 1999).

The third endosperm type, the chalazal endosperm (CE) forms as a discrete multinucleate cyst at the chalazal pole of the embryo sac and is closely associated with the maternal chalazal proliferating tissue (CPT). The CE has dense cytoplasm packed with endoplasmic reticulum (ER), dictyosomes, vacuoles, nuclei and plastids (Mansfield and Briarty, 1993). The cyst forms a distinct apical dome with a basal branched foot that has short tentacle-like protrusions, which penetrate the maternal tissue. A number of multinucleate nodules also form in this region. The location of the CE, and its high cytoplasmic activity suggests an important role in the uptake and processing of maternal resources for the developing seed (reviewed in Nguyen et al., 2000).

Further specializations of the endosperm have been described in many species. In cereals, endosperm cells in either the chalazal or/and micropylar poles form into transfer cells characterized by numerous projections into maternal tissue (Vijayaraghavan and Prabhakar, 1984; Nguyen et al., 2000). Such haustorium presumably provide a large surface area for nutrient transfer into the developing seed.

Cellularization of the endosperm begins within the ME and coincides with the initiation of cotyledons in the embryo (Mansfield and Briarty, 1993). A wave of cellularization spreads through the PE towards the chalazal pole. The NCDs polarize and form anticlinal walls, perpendicular to the wall of the central cell. The nuclei lining the central cell wall replicate and periclinal walls are formed. The process continues until the whole central cell is packed with a honeycomb of cellularized endosperm. The wave of cellularization ceases abruptly at the adaxial ridge separating the micropylar and chalazal poles. In *A.thaliana* the CE remains syncytical until the late stage of seed maturation (Mansfield and Briarty, 1990). At the onset of endosperm cellularization the developing embryo begins to absorb the endosperm and presumably assimilate the contents of the cells. A number of experiments suggest that breakdown of endosperm cells is a result of programmed cell death, perhaps triggered by high levels of ethylene (Young et al., 1997).

In cereals endosperm development follows the same 4 phases as described above. At cellularization the endosperm cells differentiate into tissue types such as starchy endosperm and aleurone, which act as storage reserves and provide hormones thought to regulate embryo growth (Olsen, 1998). In contrast to the situation in dicots, in monocots the endosperm persists until germination, and constitutes the bulk of the grain.

In summary, the endosperm is thought to act as a specialized structure facilitating the transfer of nutrients to the developing embryo. In this respect the endosperm can be seen as comparable to the mammalian placenta in its function. The 2 systems are also similar in that both the placenta and the endosperm have a maternal and paternal genetic contribution, which has dramatic implications on the development of these tissues with respect to genomic imprinting.

1.3. Genomic imprinting

1.3.1 Genomic imprinting in mammals

Genomic imprinting is the term used to describe the differential expression of genes dependent upon their parent-of-origin. To date genomic imprinting has been noted in organisms as diverse as placental mammals (Surani et al., 1990; Bartolomei and Tilghman, 1997) flowering plants (Kermicle and Alleman, 1990; Haig and Westoby, 1991) marsupials (Killan et al., 2000; 2001) and insects (Lloyd et al., 1999; Lloyd, 2000).

The initial observation that female and male genomes may be functionally non-equivalent originated from oocyte activation (Kaufman et al., 1977) and pronuclear transplantation studies in mice (Barton et al., 1984; Surani et al., 1984; McGrath and Solter, 1984). Parthenogenetic embryos, derived by the activation of an oocyte and therefore containing only a maternal genome, fail to develop to maturity (Kaufman et al., 1977). Gynogenetic embryos, formed by the nuclear transfer of two female gamete nuclei, and androgenetic embryos derived from the fusion and transfer of two male gamete nuclei into an enucleated activated egg, also eventually result in abortion (Barton et al.,1984; Surani et al.,1984; McGrath and Solter, 1984). However, these embryos exhibit distinct parent-of-origin effects. Parthenogenetic and gynogenetic mice have a well-developed embryo proper, but the extraembryonic tissue (placenta) is poorly developed or even absent. In contrast, the extra embryonic tissue of androgenones is well developed although the growth of embryos is severely retarded.

Thus a normal balance of parental genomes is essential for mammalian embryo development and the addition of extra maternal or paternal genomes has the reciprocal effect on the development of the nutrient transfer tissue (placenta). Namely, extra maternal genomes (in parthenogenic and gynogenic embryos) resulted in the poor development of the nutrient transfer tissue (placenta) while additional paternal genomes (in androgenic embryos) had the reverse effect. This reciprocal effect is due to the genomic imprinting of loci, such that the maternal and paternal genomes contribute different sets of active genes to the progeny and placental tissues.

In contrast to mammalian development many plant embryos are viable with a uniparental genetic contribution. For example, a small proportion of flowering plants are able to produce clonal, asexual seed by the process of apomixis (reviewed in van Dijk and van Damme, 2000). Common natural apomicts include dandelion (*Taraxacum*), hawkweed (*Hieracium*) and buttercup (*Ranunculus*) (reviewed in Vinkenoog et al., 2002). In apomicts the embryo is able to develop without fertilization, and thus is solely derived from maternally inherited genomic material and therefore is equivalent to the mammalian parthenogenetic embryo. In a few apomicts, mainly of the *Asteraceae* family, the endosperm is also able to develop without sexual fertilization (autonomous endosperm development). However, the vast majority of apomictic species are pseudogamous. In these plants fertilization of the central cell polar nuclei is essential for endosperm and consequently seed development. The strict requirement for both a maternal and paternal contribution to the endosperm in these plants will be discussed later in this Chapter.

Plants can also be derived artificially from cells containing a paternal genome alone using anther tissue culture. Pollen cells, when cultured on appropriate media, can develop into haploid embryoids, which can eventually grow into plants. In most species that have been tested the paternally derived plants are formed from the generative nucleus of the immature pollen grain. Examples include commercially interesting species such as; oil seed rape (*Brassica napus*) (Binarova et al., 1997), maize (*Zea mays*) (Pretova et al., 1993), tulip (*Tulipa gesneriana*) (van den Bulk et al., 1994), wheat (*Triticum aestivum*) (Ouyang et al., 1973) and barley (*Hordeum vulgare*) (Clapham, 1971). A few examples have also been recorded where androgenetic plants are apparently derived from the sperm and include henbane (*Hyoscamus niger*) (Raghavan, 1976) and carrot (*Dacus carota*) (Tyukavin et al., 1999).

Thus angiosperm embryos can develop cells containing a uniparentally-derived genome, strongly indicating that genomic imprinting has little direct impact on embryo development in plants. However, the balance of genetic contributions to the endosperm does have a strong effect on endosperm and seed development.

1.3.2 Genomic imprinting in flowering plants

The first indication that genomic imprinting may exist in plants came from the study of seed from reciprocal crosses between plants with different ploidy levels (interploidy crosses) (reviewed in Haig and Westoby, 1991). In most cases, crosses between plants of different ploidies, even of the same species results in the production of aborted seed. Often these seed have different phenotypes depending on which parent contributed more chromosome sets, with the most dramatic differences observed in the development of the endosperm. In general, high ploidy x low ploidy crosses give seed with a small endosperm, which cellularizes early, whilst the reciprocal cross gives seed with a large endosperm. Combining these observations with the proposed nutritive role of the endosperm strongly suggests that endosperm failure is the major contributing factor to seed abortion in interploidy crosses (Thompson, 1930; Watkins, 1932; Muntzig, 1933; Cooper and Brink, 1945; Brink and Cooper, 1947; Woodell and Valentine, 1961; Kermicle and Alleman, 1990; Haig and Westoby, 1991; Birchler, 1993).

A number of hypotheses have been proposed to explain the results from interploidy crosses. In the 1930s Muntzing suggested that the ploidy balance between maternal : endosperm : embryonic tissues was the critical factor for seed development, with any deviations from this 2:3:2 ratio resulting in seed abortion (Muntzig, 1930 and 1933).The ploidy of the maternal tissue was later shown to be unimportant and thus the theory that it is the endoperm:embryo genome ratio that is essential for seed development was born (Watkins, 1932; Howard, 1939). Further experiments finally provided clear evidence that the critical factor is the maternal:paternal ratio of genomes in the endosperm (reviewed in Vinkenoog et al., 2002). Perhaps the best evidence for this theory is the work reported by Lin, 1984, using the *indeterminate gametophtye* (*ig*) mutation in maize. Plants carrying this mutation can produce female gametophytes with additional polar nuclei in the central cell. By crossing *ig/ig* female plants with both 2x and 4x male plants he produced seed with 2x and 3x embryos, combined with a range of different endosperm karyotypes. 3x and 6x endosperms produced viable seed, but only when the maternal to paternal ratio was 2:1 (2m:1p). The author proposed that the 2m:1p requirement was due to the involvement of parentally imprinted genes in the development of the endosperm.

1.3.3 Parent-of-origin effects on seed development in *A.thaliana*

Reciprocal crosses between 2x and 4x *A.thaliana* plants provided the first opportunity to study endosperm development in such crosses from fertilization to seed maturity (Scott et al., 1998). In contrast to a previous report (Rédei, 1964), these crosses gave a high percentage of viable seed in both directions. Furthermore, the seed had different and complementary phenotypes with respect to mature weight and the development of the endosperm. This is despite the fact that both crosses give rise to a triploid embryo. A [2x X 4x] cross gave larger and heavier seed than a [2x X 2x] cross, while a [4x X 2x] cross gave significantly lighter and smaller seed. The endosperm of [2x X 4x] seed was over proliferated compared to that of a balanced [2x X 2x] cross. The PE exhibited accelerated mitosis and delayed cellularization resulting in an excess of endosperm cells of about the same size as those found in endosperm produced by balanced crosses. The CE was also over proliferated, with a large vacuolated cyst and many chalazal nodules. In contrast, seed from [4x X 2x] crosses showed under proliferation of the endosperm compared to balanced crosses. The PE cellularized prematurely, producing relatively few enlarged PE cells. The CE was also severely reduced in size.

Crosses between 2x and 6x plants produced inviable seed with even more extreme parent-of-origin effects on endosperm development. In [2x X 6x] seed the PE divided rapidly and was never seen to cellularize. The CE cyst and nodules also became extremely overgrown, often filling the seed at later stages of development. Conversely, the PE of [6x X 2x] seed cellularized even earlier than in [4x X 2x] seed and the CE remained small.

As Lin (1984) had previously concluded with the maize experiments, Scott et al., (1998) also suggested that these complementary phenotypes are indicative of the role of genomic imprinting in endosperm development. Furthermore, these authors proposed that the results support the parental-conflict theory of genomic imprinting (Haig and Westoby,1989, 1991; Moore and Haig, 1991).

1.3.4 The parental conflict theory of genomic imprinting

The parental conflict theory, (Haig and Westoby, 1989, 1991; Moore and Haig, 1991) although highly debated, remains the most widely accepted explanation for the evolution of genomic imprinting. According to this model, genomic imprinting arose due the competition between parental genomes over resource allocation to developing offspring. The development of seeds within a pod will be used here to illustrate the theory.

The maternal parent is equally related to all the seed produced, whilst there might be multiple paternal parents, even within a single pod. The maternal parent pays a higher reproductive cost, as she provides nutrients to the developing embryo, probably through the specialized endosperm tissues (Haig and Westoby, 1991; Lopes and Larkin, 1993; Berger, 1999; Nguyen et al., 2000). On the other hand, the sole contribution of the paternal parent to the development of the seed is his genome. As both parents contribute genetically to the endosperm (2m:1p) this allows both sets of genomes to manipulate the development of this nutrient tissue, thus maximizing their own reproductive fitness.

In the model, since the maternal parent is equally related to all the offspring, her fitness is maximized when her finite resources are distributed equally among them. As there may be multiple fathers, even within the offspring of a single pod, the fitness of the paternal parent is maximized when resources go preferentially to his offspring, regardless of the effect on other siblings. Therefore the paternal parent manipulates the allocation of maternal resources to maximize the proportion directed to his progeny.

Hence the model predicts that maternally and paternally derived alleles will have opposite effects on endosperm growth. Genes that promote the development of the endosperm are accordingly silenced when inherited from the maternal parent, but active if derived from the paternal parent. The opposite is true for genes that inhibit the development of the endosperm. In this case, maternally inherited alleles are active, and paternally derived alleles are silenced.

Thus increasing the paternal genomic contribution into the endosperm adds extra doses of active endosperm-promoting alleles, whilst extra maternal genomes provide an excess of active endosperm-limiting alleles. The results from the plant interploidy crosses support this prediction. For example, in *A.thaliana*, an excess of paternal genomes (e.g. in a [2x X 4x] cross) produces seed with an over proliferated endosperm, whilst the opposite is observed where there is an excess of maternal genomes in the endosperm (e.g. in a [4x X 2x] cross).

These results are also in accord with those from similar experiments in mice (see Section 1.3.1). Here additional maternal genomes (in parthenogenic and gynogenic embryos) resulted in the poor development of the nutrient transfer tissue (placenta) while additional paternal genomes (in androgenic embryos) had the reverse effect. Not only does this provide further evidence in support of the parental-conflict theory of imprinting but also highlights the similarity between the consequences of genomic imprinting in the animal and plant kingdoms.

However, the fact that *A.thaliana* exhibits genomic imprinting would not be predicted by the parental-conflict theory, as *A.thaliana* is a predominantely inbreeding species (Abbot and Gomes, 1989). About 98% of the time an individual plant acts as both the maternal and paternal parent and therefore both parents would bear the same cost for the progeny. It is probable however that *A.thaliana*, like other inbreeding species, evolved from an outcrossing ancestor (Stebbins, 1974). Scott et al., (1998) suggested that *A.thaliana* had retained the genomic imprinting system inherited from these outcrossing relatives, but that its effects have become attenuated over time, permitting the development of seed with limited amounts of maternal and paternal excess. It has also been argued that the complete loss of imprinting can be very difficult (Moore and Mills, 1999).

1.3.5 Genes subject to imprinting in plants

To date over 120 imprinted genes have been identified in humans, mice, rats, sheep and marsupials (Morison et al., 2001; The imprinted gene and parent-of-origin database, 2002), and have provided excellent tools with which to study genomic

imprinting. Yet very few imprinted genes have been isolated in plants. In maize, 4 loci, with a variety of functions, have been shown to be uniparentally transcribed. These are the *R* gene, which encodes a transcription factor active in the regulation of anthocyanin biosynthesis, the *delta zein-regulator* (*dzr*), a storage protein regulator, a *zein* gene and an *alpha tubulin* gene (Kermicle and Alleman, 1990; Chaudhuri and Messing, 1994; Lund et al., 1995; reviewed in Matzke and Matzke, 1993; Martinenssen, 1998).

Only a single imprinted gene, *FIS1* (Fertilization *Independent Seed-1*) ***MEDEA/EMB173*** (Peacock et al., 1995; Chaudhury et al., 1997; Grossniklaus et al.,1998), has been identified in *A.thaliana*, although there are other good candidates, including ***FIS2*** (Peacock et al., 1995; Chaudhury et al., 1997) and *FIS3/**FIE*** (Fertilization *Independent Endosperm*)(Peacock et al., 1995; Ohad et al., 1996; Chaudhury et al., 1997). Some mutant alleles of *MEA*, *FIS2* and *FIE* confer a degree of autonomous endosperm development, even in the absence of fertilization, indicating that one of the functions of these genes is to prevent endosperm development in the female gametophyte in the absence of fertilization. (Ohad et al.,1996; Chaudhury et al.,1997; Ohad et al.,1999; Kiyosue et al.,1999; Luo et al.,1999). Fertilization of ovules carrying a mutant allele results in seed abortion, even if the pollen parent provides a wild type copy of the gene. One possible explanation for this is that the genes are not active (imprinted) when inherited from the paternal parent.

A number of studies have provided strong evidence for the paternal imprinting of the *MEA* locus, although some details are contradictory. Kinoshita et al., (1999) showed imprinting of *MEA* in the endosperm, whilst Vielle-Calzada et al., (1999) showed imprinting of the locus in both the embryo and endosperm, using allele specific reverse transcriptase-PCR analysis (RT-PCR). A third group provided further evidence that *MEA* is imprinted using *MEA* promoter GUS transgenes (Luo et al., 2000). *MEA::GUS* fusion proteins were expressed in the gametophyte and in the developing endosperm of fertilized seed. However, if the *MEA* transgene was inherited from the paternal parent no GUS protein was produced during early seed development. However, *MEA::GUS* expression was noted in the chalazal cyst and

sometimes in the embryo itself in seed 2DAP. This would suggest that the imprinting of MEA is primarily in the endosperm.

Luo et al., (2000) also conducted similar promoter-reporter transgene experiments with the other *FIS* complex genes, *FIS2* and *FIE*. As with *MEA*, both transgenes were expressed in the ovule and developing endosperm (*FIE* also showed expression in some sporophytic tissues), but no GUS protein was produced in early stage seed when the transgenes were delivered by the pollen. This suggests that *FIS2* and *FIE* may also be subjected to paternal imprinting. So what are the mechanisms that could be controlling this parent-of-origin specific expression?

1.4 The mechanisms of genomic imprinting

1.4.1 Epigenetic regulation

The fundamental principle of genomic imprinting is that alleles are expressed in a parent-of-origin specific manner. As maternal and paternal alleles can be identical at the nucleotide level imprinted loci must be subjected to differential epigenetic modification to allow this parent-specific expression.

In eukaryotes approximately 146 base pairs of DNA wrap around a central core of histone proteins to form a nucleosome, the basic repeating unit of chromatin. Epigenetic modification of chromatin, such as the addition of methyl groups to cytosine residues and histone tail modifications, can result in the remodeling of this dynamic structure, facilitating or inhibiting the action of the transcriptional machinery. Both DNA methylation and histone modification have been attributed roles in mammalian imprinting (Li et al., 1993, 1994; Pedone et al., 1999; Svensson et al.,1998; Grandjean et al.,2001) .

1.4.2The methylation of DNA

The methylation of DNA bases is a modification observed in a large number of prokaryotic and eukaryotic genomes, ranging from bacterial to human, and is often associated with the repression of gene expression (reviewed in Razin, 1998).

Nucleotides can be modified by the addition of a methyl group to either adenine or cytosine residues. The reaction is under the control of a group of enzymes, collectively known as the DNA methyltransferases, which catalyze the transfer of a methyl group from the co factor S-adenosyl-L-methionine (SAM) to the correct position on the nucleotide (Billen, 1968; Lark, 1968). As the modifications that result in N4-methylcytosine and N6-methyladenine are extremely rare in eukaryotic species they will not be reviewed here. In contrast, the modification of cytosine to C5-methylcytosine is relatively abundant in many eukaryotic genomes.

All prokaryotic DNA methyltransferases share a common structure of ten conserved protein motifs (I to X) arranged in a specific order (Som et al., 1987; Lauster et al., 1989; Posafi et al., 1989). Motifs I and X are fundamental in the binding of the SAM cofactor (Cheng et al., 1993) whilst motif IV harbours the conserved proline-cysteine doublet required for methyl transfer. (Chen et al., 1991; Mi and Roberts, 1992). The target recognition domain (TRD) lies in the variable region between motifs VIII and IX, and is required to direct the enzyme to the cytosine within the recognition sequence (Wilke et al., 1988).

Eukaryotic DNA methyltransferases differ in structure and size from their prokaryotic counterparts and have two domains. The C-terminal domain retains many of the methyltransferase motifs described for the prokaryotic enzymes, and often in the same sequential order (reviewed in Finnegan et al., 1998). The N-terminal domain (which varies in length between enzymes) is fused to C-terminal region by a series of glycine and lysine repeats and contains a number of functional domains including a nuclear localization sequence (NLS) (Bestor and Verdine, 1994) and the replication foci region (Leonhardt et al., 1992; Liu et al., 1998).

1.4.3 The role of DNA methylation in mammalian imprinting

The first evidence that DNA methylation may have a role in mammalian imprinting came from experiments with mice that were homozygous for a targeted knockout of the *DNMT1* methyltransferase gene (Li et al., 1992, 1993). These embryos showed a reduction in genomic cytosine methylation of up to 70% and exhibited the loss of monoallelic expression of the 3 imprinted genes tested: *H19*, *Igf2* and *Igf2r*. However,

in contrast to the commonly attributed role of methylation in the repression of gene expression, it was found that only *H19* became biallelically expressed, whilst *Igf2* and *Igf2r* were silent. Subsequent analysis has shown that the silencing of these latter 2 loci by hypomethlyation is actually indirect, as the methylation of cis-linked sequences is involved in the regulation of their activity (reviewed in Tilghman, 1999). Nevertheless it highlights the important fact that expression is not necessarily the default state of an imprinted locus.

Following these initial experiments almost a decade of analysis has begun to unravel the role of methylation in mammalian genomic imprinting. Almost all imprinted genes studied to date contain areas of parent-specific methylation termed differentially methylated regions DMRs (reviewed in Mann et al., 2000). Removal of these regions (Thorvaldsen et al., 1998) or the loss of their parent-specific methylation (Li et al., 1992, 1993; Caspary et al., 1998) results in a marked disruption of monoallelic expression.

The DMRs are thought to act in a number of complex ways. For example, the monoallelic expression of *Igf2r* is controlled via an antisense transcript, which itself is regulated by a differentially methylated germline imprint, located in an intron (Lyle et al., 2000). On the maternal allele, the antisense transcript is repressed by methylation of the DMR, allowing transcription from the *Igf2r* promoter. The *Igf2-H19* region illustrates another type of regulation. These reciprocally imprinted loci are regulated by a differentially methylated intergenic imprinting centre (IC) (reviewed in Mann et al., 2000). The IC is unmethylated in the maternal allele and binds a methylation sensitive factor, CTCF. This prevents downstream enhancers interacting with the upstream *Igf2* promoter and results in the expression of *H19*. Methylation of the paternal IC prevents binding of CTCF allowing the expression of *Igf2*

Thus DNA methylation plays an important and complex role in mammalian imprinting and it is strongly suspected that DNA methylation acts as the primary epigenetic imprinting mark, (although it may not be the primary imprinting mechanism) (reviewed in Mann et al., 2000). However, as with every good rule there appears to be exceptions. The monoallelic expression of the *achaete-scute complex homologue-like (Drosophila)* (*Ascl2*, previously termed *Mash2*) imprinted gene is not

affected in hypomethylated *Dnmt-/-* embryos and no DMRs have yet been described for this locus (Caspary et al., 1998; Tanaka et al., 1999). Although a role for DNA methylation in the imprinting of this locus can not be ruled out, other chromatin modifications, such as histone acetylation, could be involved in the regulation of this gene (the significance of chromatin remodeling will be discussed later in this Chapter).

The enzymes involved in establishing and maintaining imprinting-associated methylation patterns are slowly being uncovered. To date, 3 functional DNA methyltransferases have been identified in the mouse: Dnmt1, Dnmt3A and Dnmt3B (reviewed in Bestor, 2000). Dnmt1 is thought to be principally a maintenance methyltransferase due to its strong preference for hemi-methylated DNA (Yoder et al., 1997). Other evidence suggests that Dnmt3A and Dnmt3B are *de novo* methyltransferases (Okano et al., 1999). Early experiments with *Dnmt1* knockout embryos suggested that Dnmt1 plays an important role in imprinting (Li et al., 1992, 1993). However, both mouse oocytes and preimplantation embryos lack Dnmt1 but express a shorter isoform of the protein, Dnmt1o (Mertineit et al., 1998). Fascinatingly, a recent report studying mice deficient for Dnmt1o suggested that this isoform is transiently required for the maintenance of methylation specifically at imprinted loci only during the fourth embryonic S phase (Howell et al., 2001). As this is the only isoform of Dnmt1 present in the preimplantation embryo this suggests that there must be another enzyme responsible for the maintenance of methylation-based imprints prior to implantation. Dnmt3A and Dmnt3B are considered to be unlikely candidates due to their predicted *de novo* function (Okano et al., 1999) and the fact that patients with ICF syndrome, caused by mutations in *DNMT3B*, retain allele specific methylation at the *H19* locus (Xu et al., 1999). Thus it remains a strong possibility that other murine DNA methyltransferases have yet to be identified.

Therefore, in mammals, DNA methylation patterns established and propagated by a number of enzymes play an essential role in genomic imprinting. In flowering plants the mechanisms of parental imprinting are far less clear. However strong circumstantial evidence suggests that DNA methylation may also have an important part to play.

1.4.4 DNA methylation in flowering plants

The genome of plants, as is mammals, exhibits significant levels of DNA methylation. Indeed in species such as rye 33% of the cytosines are modified (Thomas and Sherratt, 1956). Methylation in plants also occurs commonly at both CpG and CpNpG sites (where N is any base) and at non-symmetrical sites, (Gruenbaumen et al., 1981; McClelland, 1983; Meyer et al., 1994) in contrast to mammals where methylation is restricted primarily to CpG sequences (Bestor, 1993).

The first clue that DNA methylation may be important for normal plant development came from the treatment of seeds and plant tissue cultures with the chemical 5-azacytidine (5-azaC) which causes a reduction in the level of DNA methylation. Plants grown from seed treated with 5-azaC were shown not only to contain hypomethylated DNA, but also to have heritable phenotypic changes in development (Sano et al., 1990; Heslop-Harrison, 1990; Fieldes, 1993). In rice and flax the predominant phenotypic effect was a reduction in plant height (Sano et al., 1990; Fieldes, 1993). In *Triticale* the treatment induced a number of other changes including an increase in plant height, a decrease in the age to maturity and an increase in tillering (Heslop-Harrison, 1990). Treatment with 5-azaC was also shown to induce early flowering, mimicking the developmental process of vernalization (Burn et al., 1993; Brock and Davidson, 1994). Such heritable changes in development suggested that DNA methylation has a role in regulating gene expression and therefore could also regulate the expression of imprinted plant genes.

More direct evidence to suggest that DNA methylation could have a role in the regulation of genomic imprinting in plants came from the study of the imprinted genes in maize. Expression of imprinted *zein* genes is restricted to the endosperm when inherited from the maternal parent, and these alleles are hypomethylated compared with the paternally inherited allele (Lund et al., 1995). The *R* locus also shows differential methylation patterns that are dependent on its parent-of-origin (Kermicle and Alleman, 1990; Finnegan et al., 1998). Another clue indicating the potential role of DNA methylation in genomic imprinting came from the rapidly expanding field of plant DNA methyltransferase research.

The first plant DNA methyltransferase, *MET1*, was identified in *A.thaliana* (Finnegan and Dennis, 1993). Since then *MET1* homologues have been identified in carrot (Bernacchia et al., 1998), maize (Olhoft, 1998), pea (Pradhan et al., 1998) and tobacco (Nakano et al., 2000). All encode proteins with a structure that is strikingly similar to the murine Dnmt1 enzyme.

The highest level of conservation between the *A.thaliana* MET1 and Dnmt1 is within the methyltransferase domains (50% amino acid identity) with homology dropping to 24% identity in the N-terminal region (Finnegan and Dennis, 1993; Finnegan and Kovac, 2000). Indeed, in general the mammalian and plant enzymes exhibit a number of distinct differences in potentially functional regions within the N-terminal. For example, mammalian methyltransferases have a cysteine-rich zinc-binding region motif (Bestor, 1992), which is absent in the plant protein. Furthermore, the plant MET1 class enzyme has a distinct acidic region that is not observed in the mammalian proteins (Finnegan and Dennis, 1993; Genger et al., 1999).

Nevertheless, despite these differences the degree of homology within specific regions of the N-terminal suggests the MET1class of enzyme may have a similar role to Dnmt1. This region localizes the mammalian enzyme to the nucleus (Bestor and Verdine, 1994) and during S-phase targets it to the replication fork (Leonhardt et al., 1992; Liu et al., 1998). The N-terminal also contains motifs that enable the enzyme to distinguish between hemimethylated and unmethylated DNA, resulting in a strong preference for a hemimethylated template (Bestor, 1992). This not only strongly suggests that the METI enzymes function as maintenance methyltransferases but additionally that they could play a similar role to Dnmt1 in the regulation of genomic imprinting in plants. However, as with mammals, DNA methylation, and therefore by inference genomic imprinting, may be under the control of a number of different DNA methyltranferases.

1.4.5 Plant DNA methyltransferases

In the completed *A.thaliana* genome a total of 9 putative DNA methyltransferases have been identified (Finnegan et al., 1993; Heinkoff and Comai, 1998; Genger et al.,

1999; Cao et al., 2000; Finnegan and Kovac, 2000 and McCallum et al., 2000). These putative enzymes have subsequently been classified by homology into three separate groups: the Methyltransferase 1(MET1) class, (Finnegan et al., 1993; Genger et al., 1999) the Chromomethylases (CMT) class (Heinkoff and Comai, 1998) and the de novo DNA methyltransferase class (Cao et al., 2000). Evidence is mounting to suggest that each group of enzymes may have a distinct role to play in the methylation of the plant genome.

1.4.5.1 MET1 DNA methyltransferases

In *A.thaliana MET1* is a member of a small gene family, all members of which are thought to have arisen from the same ancestral gene (Finnegan and Kovac, 2000). The other members: *METIIa*, *METIIb* and *METIII* are expressed at much lower levels than *MET1* (Genger et al., 1999). To date, experiments to elucidate the role of *METIIa* and *METIIb* have proved unfruitful (J.Finnegan personnal communication). However, there is evidence to suggest that *METIII* encodes a nonfunctional protein in the ecotype Col suggesting it has a non-essential role in development (Genger et al., 1999).

The role of MET1 in DNA methylation and plant development as a whole has been extensively studied using *A.thaliana* lines carrying a *MET1* antisense transgene (*MET1a/s*) (Finnegan et al., 1996; Ronemus et al.,1996). These plants exhibited significant levels of demethylation in both single-copy and repeated DNA sequences. This demethylation occurred preferentially at CpG sequences, leading to the suggestion that the primary function of MET1 class enzymes is to maintain methylation at CpG sites (Finnegan and Kovac, 2000).

The hypomethylated transgenic plants also developed a number of abnormalities including the formation of aerial rosettes, a greater number of cauline leaves, alteration in flowering time, secondary inflorescences on the primary bolt and homeotic changes to flower structure that resemble those caused by floral homeotic mutations. This suggests that MET1 activity is required for normal development. Indeed several genes, including the floral regulatory loci *APETALA3* (*AP3*) and *AGAMOUS (AG)*, were ectopically expressed in the leaves of *MET1a/s* plants

(Finnegan et al., 1996) suggesting MET1 catalysed methylation acts to repress gene expression. Unexpectedly, *AG* and another floral regulatory gene *SUPERMAN* (*SUP*) were silenced in some mutant flowers of *MET1a/s* plants. Furthermore, the silent state of these loci correlated with **hyper**methylation in the *AG* gene and in a region upstream of the *SUP* gene (Jacobsen and Meyerowitz, 1997; Jacobsen, 1999; Jacobsen et al., 2000). Recent reports have now linked this hypermethylation to another methyltransferase, chromomethylase 3 (CMT3) (Lindroth et al., 2001), highlighting the complex nature of plant DNA methylation.

1.4.5.2 Chromomethylases

The first chromomethylase, (*CMT1*), was identified in *A.thaliana* (Heinkoff and Comai, 1998) and a further 2 genes (*CMT2* and *CMT3*) have since been isolated (Finnegan and Kovac, 2000; McCallum et al., 2000). CMT encoding genes have also been found in plant as diverse as *Brassica* and maize, but perhaps significantly none have been identified in a species outside of the plant kingdom (Rose et al., 1998; Finnegan and Kovac, 2000).

The CMT proteins are distinctly different in structure to the MET1 class of DNA methyltransferases. The CMT enzymes have an N-terminal region that is approximately 750 amino acids shorter than the MET1proteins and are characterized by the presence of a chromodomain motif between the cytosine methyltransferase catalytic domains I and IV (Heinkoff and Comai, 1998). As chromodomains are thought to mediate interactions between chromatin components and are proposed to function in the formation of heterochromatin, this makes CMT enzymes interesting candidates for a role in plant genomic imprinting (reviewed in Paro and Harte, 1996).

In many *A.thaliana* ecotypes the coding sequence of CMT1 is interrupted by the insertion of the Evelknievel retrotransposon (Heinkoff and Comai, 1998) leading to the production of a truncated CMT1 protein suggesting the *CMT1* gene encodes a non-essential enzyme. In contrast, *CMT2* and *CMT3* appear to encode functional proteins. Recent studies have suggested that the CMT3 methyltransferase (and the maize homolgue, *Zea methyltransferase 2*) are required for the maintenance of

CpNpG methylation patterns in the plant genome (Bartee et al., 2001; Lindroth et al., 2001; Papa et al., 2001).

A.thaliana plants carrying *cmt3* loss-of-function alleles exhibited reduced methylation at the site CpNpG. (Bartee et al., 2001a,b ; Lindroth et al., 2001). Little, if any, reduction of methylation was seen at CpG sites although at some loci the methylation of cytosines at non-symmetrical sites was also affected. Lines carrying mutant *cmt3* alleles also showed the reactivation of a subset of endogenous retrotransposons, suggesting that CMT3 is required for maintaining silencing at these loci. Interestingly, in contrast to hypomethylated *MET1a/s* plants, plants carrying the *cmt3* mutation do not show developmental abnormalities, even after several rounds of inbreeding.

A similar situation was observed in maize, where plants carrying a *Mutator* transposable element in the chromomethylase gene *Zmet2,* also showed specific demethylation of CpXpG sites, with no corresponding reduction in CpG methylation (Papa et al., 2001). Furthermore, and in contrast to the experiments with *MET1a/s A.thaliana* plants, the progeny of demethylated maize plants showed remethylation upon segregation away from the mutated *Zmet2* locus. Whether ZMET2 has a *de novo* function, allowing the re-establishment of methylation, or whether another methyltransferase sets up the methylation marks and ZMET2 is subsequently required for the maintenance of the methylation patterns, remains to be determined.

The function of the CMT2 gene product in *A.thaliana* as yet remains unknown. If *cmt3* plants are transformed with wild type CMT2, the *cmt3* phenotype (reduced CpNpG methylation) is not rescued, suggesting that CMT2 cannot substitute for CMT3 function (Bartee et al., 2001). Interestingly the two proteins differ primarily in their N-terminal sequence, suggesting that that they may interact with different targets.

Therefore, the CMT family of methyltransferases is required for the methylation of cytosine at the recognition site CpNpG and possibly also at asymmetric sequences. One possibility is that the CMT proteins act in some way (probably via the conserved chromodomain) to promote the formation of heterochromatin. The packaging of DNA into a stable silent state is fundamental to the process of genomic imprinting and

therefore the potential role of CMT proteins in plant genomic imprinting is an exciting prospect.

1.4.5.3 The *de novo* DNA methyltransferases

The third group of plant DNA methyltransferases was identified via their high degree of sequence identity to the *Dnmt3* class of methyltransferases (Cao et al., 2000). *Dnmt3* genes have been found in mouse, human and zebrafish (Okano et al., 1998; Xie et al.,1999) and are strongly suspected to encode *de novo* methyltransferases. For example murine recombinant Dnmt3a and Dnmt3b enzymes show *de novo* activity on unmethylated DNA *in* vitro (Okano et al., 1998). Furthermore when Dnmt3a is expressed in *D.melanogaster*, *de novo* methylation of the genome was observed (Lyko et al., 1999). The *D.melanogaster* genome usually has extremely low levels of methylation (Gowher et al., 2000).

To date DNA methyltransferase genes, with a high identity to the Dnmt3 class, have been characterised in *A.thaliana* and maize (Cao et al., 2000). The highest identity with these proteins and Dnmt3 lies within the methyltransferase catalytic domains (on average 28% amino acid identity). In contrast the N-terminal regions of the plant enzymes had no detectable significant similarity with the equivalent animal proteins.

The plant proteins show a novel arrangement of the 8 conserved catalytic domains in the C-terminal region. In most eukaryotic methyltransferases the motifs lie in the following order, I, II, III, IV, V, VI, IX and X, from the N-terminal region. But in the plant sequences the order of the motifs has been rearranged to, VI, IX, X, I, II, III, IV and V. The novel order of the conserved domains was also maintained in a soybean cDNA identified via its sequence homology to other plant enzymes. It has been predicted that as motifs I and X actually lie parallel to each other in the tertiary structure of the prokaryotic *Hha1* methyltransferase, the specific rearrangement of the domains may have little overall effect on the folding and function of the plant proteins (Cao et al., 2000).

Due to the novel order of the catalytic motifs the *A.thaliana* proteins were named the domains rearranged methyltransferases (DRM). Two *A.thaliana* sequences were

identified, *DRM1* and *DRM2*, but only *DRM2* has been characterised in detail. The maize sequence was named *Zmet3* and represents the third class of methyltransferase identified in this species.

The DRM2 and Zmet3 proteins also contain ubiquitin-associated (UBA) domains in their N-terminal domain. Such domains have never been observed in any other class of methyltransferase, including the animal *Dnmt3* class of enzymes. The exact function of UBA domains is unclear, although UBA containing proteins show a variety of associations with the ubiquitin pathway (Schauber et al., 1998; Wang et al.,1996; Lee et al.,1999). It is possible that the UBA domains are required for regulation of the DRM2 and Zmet3 proteins through the cell cycle via ubiquitin based degradation (Cao et al., 2000). Alternatively, ubiquitination may regulate the localization of the plant enzymes in response to such factors as external signals or transposon activity.

In summary, the third class of plant DNA methyltransferases is novel both in the arrangement of the catalytic motifs and the incorporation of UBA domains in the N-terminal domain. The high degree of sequence identity of the plant enzymes with the Dnmt3 class of methyltransferases strongly suggests that they too may act as *de novo* methyltransferases. As imprints must be set within each generation the predicted *de novo* activity of these enzymes makes them attractive candidates for a role in genomic imprinting in plants.

1.4.6 DNA methylation requires the action of proteins in addition to DNA methyltransferases

In *A.thaliana* the isolation of the *Decrease in DNA methylation 1* (*DDM1*) gene was the first indication that proteins other than DNA methyltransferases are important for DNA methylation in plants. Plants homozygous for a mutation in *DDM1* (*ddm1/ddm1*) show dramatic hypomethylation of the genome and pleiotropic effects on development, which increase in severity with each selfed generation (Vongs et al., 1993; Kakutani et al., 1996).

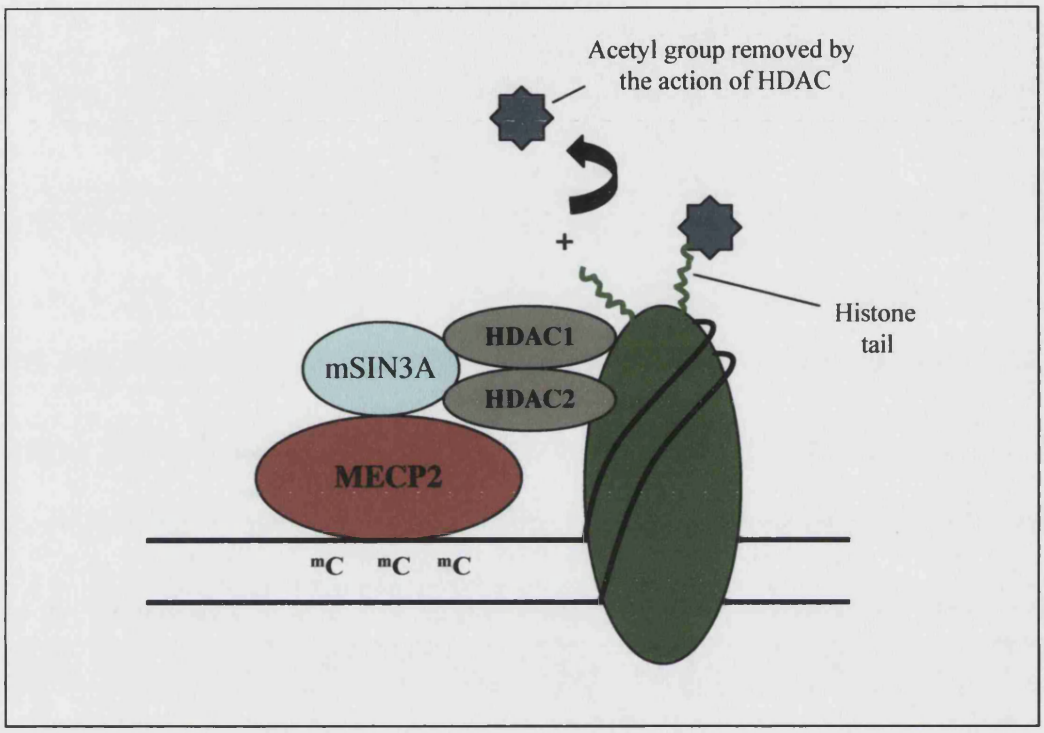
DDM1 encodes a member of the SNF2/SWI2 family of ATP-dependent chromatin remodeling proteins (Jeddeloh et al., 1999). These proteins act as complexes, binding to DNA and nucleosomes. Driven by ATP hydrolysis they disrupt nucleosome structure (reviewed in Peterson and Workman, 2000). Although the exact function of DDM1 in DNA methylation is unknown, it is likely that DDM1 remodels the chromatin allowing access of the methyltransferases to the DNA. Thus DDM1 could also play a part in regulating genomic imprinting in plants. Indeed, *ddm1/ddm1* mutants have been used to study the role of DNA methylation in genomic imprinting (Vielle-Calzada et al.,1999) and the results of these experiments will be discussed in Chapter 8.

1.4.7 The role of DNA methylation in the regulation of plant gene expression

As described previously, DNA methylation is generally associated with the repression of gene expression (reviewed in Razin, 1998). This repression can be direct, with methylation of the promoter region or control elements inhibiting the binding of factors of the basal transcriptional machinery. However, DNA methylation alone is often not sufficient to block transcription (Ng and Bird, 1999) but instead it is the formation of condensed chromatin mediated by DNA methylation which leads to silencing.

A simple schematic example is shown in Figure 1.3. Here, MECP2 (a methyl binding protein) recognizes and binds the site of methylation and recruits among other proteins, histone deacetylases (HDAC). The subsequent removal of the acetyl groups from the histone tail by the action of HDAC represses gene expression by increasing the affinity of the now positively charged lysines to the negative charge of the DNA backbone (reviewed in Rice and Allis, 2001). This remodels the chromatin into a higher order structure, which is unfavourable to transcription.

Such remodeling of the chromatin into a silent state is of particular interest with respect to genomic imprinting, as it would allow the imprinted state of an allele to be propagated through many cell divisions in the developing mammalian embryo or plant endosperm. Indeed, histone acetylation has been directly linked with



Revised from Razin, (1998)

Figure 1.3

Transcriptional repression complex. The methyl binding protein (MECP2) recognizes and binds the site of DNA methylation (^{m}C). This then recruits mSin3A to which HDAC1 and HDAC2 are associated. The HDACs then remove the acetyl groups from the lysine residues on the histone tail and the positive charge of the lysine increases the affinity of the histone to the DNA, forming a stable repressed higher order chromatin structure.

mammalian imprinting. In one study, Grandjean et al., (2001) showed that both the *H19* and *Igf2* genes exhibit parent-of-origin specific acetylation patterns in their promoter regions, with hyperacetylation correlating with hypomethylation on the active allele. A second group showed that treatment with a HDAC inhibitor, trichostatin A (TSA), could reactivate the normally silenced *Igf2* maternal allele after about 24h (Pedone et al., 1999) although interestingly no effect was seen on the paternal *H19* and *Igf2* alleles after a similar treatment with TSA (Grandjean et al.,2001). Nevertheless, the importance of histone acetylation in mammalian imprinting is now becoming apparent.

Histone acetylation also plays an important part in the regulation of higher order chromatin structure in plants. A large number of histone acetyltransferases (HAT) and HDAC have now been identified in plants (reviewed in Finnegan, 2001; Chromatin database, 2002) and current research is beginning to unravel their role in development. *A.thaliana* plants with reduced activity of the HDACs AtHD1/AtRPD3A or AtHD2 show a number of abnormal phenotypes including seed abortion, early seedling lethality, reduced apical dominance, floral homeotic transformations and early senescence (Wu et al.,2000a; 2000b; Tian and Chen, 2001; reviewed in Finnegan, 2001). Not only does this show that histone acetylation is required for normal plant development, but also highlights the potential role of histone acetylation in the regulation of plant genomic imprinting.

The acetylation of histone tails is only one of the post-translational modifications that can result in changes in gene expression. Modification by methylation, ubiquitination and poly (ADP-ribosylation) of histones can also regulate chromatin structure (Spencer and Davie, 1999; Wu and Grunstein, 2000; Rice and Allis, 2001). The methylation of histones is particulary interesting with respect to genomic imprinting as it is associated with the formation of heterochromain and may represent a highly stable modification for the long term shut down of gene expression (reviewed in Rice and Allis, 2001). Thermodynamically, methyl-lysine is more stable than the acetyl or phosphoryl modifications. Furthermore, whilst the two latter modfications can be removed by HDACs or phosphatases respectively, no histone demethylases have yet been identifed. It is possible histone methylation would have be removed by either proteolytic or ubiquitin-based processing of the histone tail. Such a stable epigenetic

process may well function in genomic imprinting. This hypothesis is further supported by the observation that during vertebrate spermatogenesis almost all the histones are removed, which would theoretically facilitate the resetting of imprints.

Thus the regulation of gene expression in both mammals and plants is controlled by modifications of the higher order chromatin structure. Many factors contribute to this remodeling and the exact function of the various components requires further attention. However, what is clear is that the extensively studied process of DNA methylation (and to a lesser extent histone acetylation) plays an important role in genomic imprinting in mammals.

Although genomic imprinting has evolved separately in plants and mammals the 2 systems share a number of similarities. Strikingly an imbalance of genomes in both mammals (embryo) and plants (endosperm) results in reciprocal parent-of-origin effects on the development of the nutritive tissues (the placenta and the endosperm respectively). It is also proposed that both systems arose due to parental conflict over resource allocation from the mother to her progeny (Haig and Westoby, 1989, 1991; Moore and Haig, 1991). It therefore appeared a plausible hypothesis that the regulation of genomic imprinting may also be similar in both systems. Indeed, DNA methylation in plants, as in mammals, has been shown to be important for the regulation of gene expression. Furthermore, the enzymes that catalyze methylation also show a high level of conservation between the 2 kingdoms, suggesting that they could have similar functions. Therefore we proposed to test the role of DNA methylation in genomic imprinting in *A.thaliana* and this forms the basis of most of the work described in this thesis. The main motivation behind the work came from the potential application of genomic imprinting in biotechnology.

1.5 The possible applications of genomic imprinting in plants

Discovering the genes and mechanisms involved in plant genomic imprinting would not only offer insight into this intriguing epigenetic phenomenon, but also provide us with a multitude of biotechnology applications. The advances made in recent years with respect to genetically modified (GM) crops have caused apprehension in both the scientific and public domains over the effects of their deployment. Perhaps the most

pressing concern is that modified genetic material could escape into closely related species with unforeseeable consequences. A number of confinement techniques have been explored, including the use of male sterility (Gray and Raybould, 1998) and the transformation of the maternally inherited chloroplast genome, rather than the nuclear genome (Daniell et al., 1998). However, the use of male sterility is far from ideal, especially in regions of the world where farming depends on seed collected from farm saved seed. An alternative could be to manipulate the imprinting system of the GM crops.

As described previously most species have a strict requirement for a (2m:1p) ratio in the endosperm, with any deviation from this resulting in seed abortion. According to the model proposed to account for the evolution of genomic imprinting in plants (Haig and Westoby,1989, 1991; Moore and Haig, 1991; Scott et al.,1998) the cause of seed failure is an imbalance of imprinted gene expression in the endosperm. Elucidating the mechanism of genomic imprinting could enable the design of imprinting based strategies to engineer GM crops to produce a hybridization barrier between the modified crop and its close relatives. In other words, the degree of genomic imprinting in crops could be modified such that attempted hybridization between GM plant and a wild relative for example, results in a lethal genomic imbalance within the endosperm resulting in death of the hybrid seed. Importantly, self-fertilization would result in normal seed production since such a system would preserve the usual balanced genomic ratio within the endosperm.

Another attractive prospect for the use of genomic imprinting in biotechnology is to breakdown natural hybridization barriers between different species. This would allow the production of new hybrid species with previously untapped potential. As with intra-specific crosses, successful inter-specific hybridizations require the correct balance of maternal:paternal genomes. However, the genomes of different species are not necessarily equivalent with respect to genomic imprinting, even if they are of the same ploidy. In other words, a 2m:1p ratio of genomes in the endosperm will not always result in the production of viable seed. The failure of inter-specific crosses has been well studied, particularly within the genus *Solanum*, and led to the proposal of the endosperm balance number (EBN) hypothesis. (Johnston and Hanneman, 1980)

According to the EBN hypothesis each species has a genome specific effective ploidy level, its EBN, which is not necessarily equivalent to its actual ploidy. Thus, in an inter-specific cross it is the EBN values that must be in the ratio of 2maternal: 1paternal in the endosperm to allow successful seed development. The EBN of a species can be interpreted in terms of genomic imprinting (Ehlenfeldt and Ortiz, 1995). A species with a high EBN may have a strong genomic imprinting system. This could be due to a large number of genes being subjected to parental imprinting and/or the genes having a large effect on endosperm development. The opposite may be true of species with a low EBN. If differences in EBN are due to variation in genomic imprinting strength, the manipulation of imprinting could permit the hybridization of species with different EBN values.

A further exciting possibility for the use of genomic imprinting in biotechnology is the introduction of apomixis into plants of agricultural significance. Many crop species used today are F1 hybrid species, chosen for the heterosis they exhibit compared to their parental lines. However, a significant cost and therefore limitation of F1 hybrid technology is that F1 seed must be generated annually by crossing the parental lines, as the F2 plants show hybrid breakdown. The introduction of full apomixis into F1 hybrid crops would allow the clonal propagation of seed without the loss of hybrid vigor. However, most natural apomitic species still require the polar nuclei to be fertilized by a sperm for successful seed development. This requirement for a sexual endosperm, often with a 2:1 maternal to paternal genome ratio, is thought to be due to genomic imprinting. It is possible that the manipulation of genomic imprinting could permit the development of fully apomitic crop plants.

1.6 The aim of this Thesis

As described above, the primary aim at the beginning of the work described in this Thesis was to test the hypothesis that DNA methylation plays a role in genomic imprinting in *A.thaliana*. The role of DNA methylation in mammalian imprinting was first indicated by the loss of monoallelic expression of imprinted genes in mice homozygous for a targeted knockout of the *DNMT1* locus (Li et al., 1992, 1993). At the outset of the present work, plant lines were available that exhibited a high degree of genome hypomethylation due to the expression of an antisense trangene to the main

A.thaliana DNMT1 homologue, *MET1* (Finnegan et al., 1996). However, at the time only one gene, *MEA*, was suspected to be imprinted in *A.thaliana* (Grossniklaus et al., 1998). Therefore testing directly the loss of monoallelic expression was not feasible. However, a method was available by which we could assay an imbalance of genomes, and by inference an imbalance of imprinted gene expression, by observing the development of the endosperm.

The parent-of-origin effects on the seed from reciprocal interploidy crosses in *A.thaliana* provided us with a way of measuring the effect of adding extra paternal genomes (and therefore extra imprinted genes) into the endosperm. An increase in maternal genomic contribution (for example a [4x X 2x] cross), resulted in seed with an under developed endosperm and a reduced mature seed weight. Seed with opposite phenotypes were produced by increasing paternal genomic contribution via, for example, a [2x X 4x] cross.

We set out to test the proposition that DNA methylation plays a role imprinting in plants by examining the outcome of crosses involving plants with a hypomethylated genome. For example, we predicted that if DNA methylation plays a role in the parent-of-origin effects, by conducting reciprocal crosses between hypomethylated *MET1a/s* and wild type plants we would have a parent-of-origin effect on seed development that phenocopied the addition of extra parental genomes to the endosperm (for more detail on the concept of this experiment see Section 3.1) The exciting results from these experiments are described in Chapter 3.

However, although *MET1* was a credible candidate for a role in genomic imprinting the existence of other DNA methyltransfereases kept open the possibility that imprinting could be under the control of a number of different DNA methyltransferases. Therefore a systematic search was conducted for mutations in both *MET1* and other loci encoding putative DNA methyltransferases in *A.thaliana*. The ultimate aim was to use these mutant lines in similar experiments to those described in Chapter 3.These screens are described in Chapter 4.

The work outlined in Chapter 5 was based on the preliminary observation that in crosses between hemizygous *MET1a/s* plants and 4x plants the rate of seed abortion correlated with the age of the parental plants (described in Chapter 3). In order to test if the age of parental plants has an effect on the parent-of-origin effects the crosses were repeated with plants at set developmental stages.

Little was known about the expression profiles of any of the putative DNA methyltransferases during floral development, gametogenesis or seed development. Theoretically this is the temporal window during which imprints must be set and maintained. For this reason the work outlined in Chapter 6 examines the expression pattern of 2 DNA methyltransferases (MET1 and DRM2) during these stages of development.

Finally, it was recognized that DNA methylation is probably only one potential component of the mechanism of genomic imprinting in plants. Furthermore, only one imprinted gene had been identified, despite the dramatic effect on seed development of the interploidy crosses. Therefore a screen of mutagenized seed was designed and conducted to identify genes involved in genomic imprinting in plants. The design of the screen and the characterization of one of the candidates are described in Chapter 7.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Plant material

2x and 4x *A.thaliana* plants were of either the C24 or Col ecotype. 4x C24 seed were kindly donated by Eric van der Graff (University of Leiden, Netherlands). The 4x Col seed were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC, UK). The 2x C24 plants were hemizygous for the A9-barnase transgene conferring male sterility (Paul et al., 1992); these segregate 1:1 male sterile and male fertile plants. The male sterile segregants were used as the female parents as emasculation was not required.

The *MET1a/s* seed were in the C24 background and were kindly donated by Jean Finnegan (CISRO, Australia). They were from the T3 generation of family 10.5 and were homozygous for the *MET1a/s* transgene driven by the cauliflower mosaic virus 35S promoter (Finnegan et al., 1996).

The EMS treated seed were in the Col-3 background and were obtained from LEHLE SEEDS (Round Rock, Texas, U.S.A). In total 16 different parental groups were used. Each parental group consisted of 0.11g of M2 seed from 680 ± 14 M1 self fertilized plants. The mutation rate of the original M1 population was estimated as P= 0.5, M= 0.69, according to the method described by Mendik (1998).

2.1.2 Plant growth media

Germination media (GM) was composed of 1x Murashige and Skoog (MS) salts with Gamborg B5 vitamins (Sigma, Dorset, U.K.) and 0.5g/L of 2-(N-morpholino) ethanesulfonic acid (MES). The pH was adjusted to 5.8 with NaOH. For plates the GM was solidified with 0.8% agar. Top agar consisted of 0.8% agarose.

2.1.3 Bacterial strains

Escherichia coli strains *DH5α* and *XL1-Blue* were used for cloning. For plant transformation the *Agrobacterium tumefaciens* strain used was GV3101 harbouring a non-oncogenic Ti plasmid (pGV301) (Van Larebeke et al., 1974).

2.1.4 Bacterial growth media

E.coli and *A.tumefaciens* were grown in 2xTY media containing 5g/L NaCl, 10g/L yeast extract and 16g/L bactotryptone and the pH adjusted to 7.0 where necessary. Plates were solidified with 2% bacto-agar. Liquid media or plates were supplemented with antibiotics at concentrations of 100μg/ml ampicillin, 50μg/ml kanamycin, 10μg/ml tetracycline or 20μg/ml chloramphenicol for *E.coli*, and 100μg/ml rifampicin, 25μg/ml gentamycin and 100μg/ml spectomycin for *A.tumefaciens*.

2.1.5 Plasmids and probes

Plasmids used for cloning were pGEMT (Promega, Southampton, UK), BJ60 and BJ40 donated by B.Janssen, (Hort+Research, Aukland, NewZealand). The pARR20-1 180bp centromere clone was a gift from E.Richards (Harvard University, USA). Although pARR20-1 is so far unpublished similar clones are described in Martinez-Zapater et al., (1986).

2.1.6 Oligonucleotides and sequencing

Gene specific oligonucleotides and linkers were synthesized by Life technologies. All DNA sequencing reactions were carried out by Paul Jones at the University of Bath.

2.2 Methods

2.2.1 Plant growth conditions

Seeds were sown on to F2 soil and stratified at 4°C for 4 days before being transferred to a growth room with a day length of 16 hours and temperature of 22°C. After 2 weeks seedlings were repotted in insecticide soil and transferred to a glass house and grown at 24± 2°C.

2.2.2 Cross pollinations

When the seed parent was male sterile (A9-barnase), open flowers were pollinated. Were plants male fertile, flower buds were emasculated 1 day prior to anthesis and pollinated 2 days later.

2.2.3 Seed mass analysis

Mature seeds were collected when pods were desiccated and stored until required in 1.5ml tubes with pierced lids. Seeds were then weighed, in groups of 10, using a Mettler UMT 2 microbalance (Mettler-Toledo, Leicester, U.K).

2.2.4 Confocal microscopy

Seed pods were collected and stored in 3:1 ethanol to acetic acid at 4°C until required. The samples were then stained with Schiffs reagent (Sigma) and fixed in LR white (Agar Scientific, Essex, U.K) as described by Braselton et al., (1996) and imaged using an Axiovert 100M Zeiss LSM510 laser scanning microscope (Carl Zeiss Ltd., Wellwyn Garden City, UK). The Feulgen-stained samples were excited with an argon ion laser at 458 or 488 nm, and emissions were detected at ≥515nm. Images measuring 1024 x 1024 pixels were collected with a C-Apochromat 63x/1.2 water lens (Zeiss).

2.2.5 DNA analysis

2.2.5.1 Isolation of plant DNA for PCR

DNA preparations were made on a small scale based on the method by Edwards et al., (1991). Single leaf samples were harvested, frozen in liquid nitrogen and stored at −80°C until required. The tissue was then broken up using a cooled eppendorf grinder for 20s and 400µl of PCR extraction buffer (200mM TrisHCL pH7.5, 250mM NaCl, 25mM EDTA, 0.5%SDS) added. The mixture was then vortexed for 5 s and centrifuged at 15 000Xg for 8 min. 300µl of the supernatant was then transferred to a fresh tube and an equal volume of propanol added. After a 2 minute incubation at RT the precipitate was collected by centrifugation at 15 000Xg for a further 8 mins . The supernatant was removed by careful pipetting and the pellet air dried for 10 mins prior to resuspension in 50µl of dH_20 and stored at −20°C. In most cases 4 to 5µl would then be used in a PCR reaction.

2.2.5.2 Isolation of plant DNA for enzyme digestion and Southern analysis

DNA was extracted from 0.1g of leaf tissue using a Nucleon Phytopure Plant DNA Extraction Kit (Nucleon, Biogenesis, Glasgow, UK) according to the manufacturers instructions. 100ng (approximately 5µl of the DNA prep) was used in following digests.

2.2.5.3 Preparation of bacterial plasmids

Bacterial cultures were grown from individual colonies in 5ml of 2xTY media in sterile universals overnight at 37°C, 200rpm. Cells were harvested from 1.5ml of the culture by centrifugation at 15 000g for 5 min and the supernatant removed. The plasmid DNA was then isolated using the QIAprep® Spin Miniprep Kit (QIAGEN, West Sussex, UK) according to the manufacturers instructions. 50µl of sterile dH_20 was always used in the final step and samples stored at -20°C.

2.2.5.4 Polymerase Chain Reaction (PCR)

Based on the methods by Innis et al., (1990), Taq polymerase (Promega) was used in conjunction with an 11x buffer (500mM TrisHCL pH 8.8, 120mM NH_4SO_4, 50mM $MgCl_2$, 75mM ME, 0.05mM EDTA, 11mM dNTPs, 1.25mg/ml BSA (DNAse free)). In general 10ng of template DNA was used in each reaction. Primers were used at a concentration of 10pmol. A typical 20µl PCR reaction was as follows; 3µl of DNA template, 2µl of forward primer, 2µl of reverse primer, 1.8µl of 11x buffer, 0.2µl of Taq polymerase and 11µl of dH_20. Initial annealing temperatures used were those calculated by Life Technologies. If greater specificity was required the annealing temperature was raised. Elongation time was calculated as 1 min for each kb to be amplified. All Reactions were carried out on a MJ Research PTC-200 Peltier Thermal Cycler. A typical PCR program was an initial denaturation step of 94°C for 3 min, followed by 30 cycles of 94°C (denaturing) 1 min, 50°C (annealing) 1min, 72°C (extending) 2 min.

Extensor Taq polymerase (Promega) was used for amplifying the *MET1* and *DRM2* promoter fragments for cloning according to the manufacturers instructions. As the *MET1* promoter showed no amplification with the normal Taq, extensor Taq was also used for all PCR reactions with this fragment (including colony PCR).

2.2.5.5 Colony PCR

PCR reactions were carried out as described in section 2.2.5.4, except the volume of DNA template was replaced with dH_20. A sample of colony to be screened was transferred to the PCR mixture using a pipette tip, and the reaction mixed gently. The PCR reaction was then performed as normal. The screened plate was incubated at 37°C for a further 4 hours to allow additional growth of the colony.

2.2.5.6 Agarose gel electrophoresis

Electrophoresis and agarose gel preparation was carried out using Bio-Rad trays and tanks (Bio-Rad Laboratories Ltd, Hertfordshire, UK). Gels were prepared by melting

0.8-1.5% agarose (BDH, Dorset, UK) in the required amount of 1xTAE (40mM Tris-acetate, 1mM EDTA) by boiling. 3µl of 10 mg/ml EtBr was added per 100ml of gel mix. DNA samples were mixed with loading buffer (0.25% (w/v) bromophenol blue, 0.25% Xylene Cyanole FF, 15% Ficoll type 400 in dH20) and loaded into the gel wells. Electrophoresis took place in a Sub-Cell tank (Bio-Rad) with a Bio-Rad Powerpac 300 power supply, usually at 100V. Running buffer was1x TAE. DNA bands were visualized on a transilluminator with UV light at 254 nm.

2.2.5.7 Purification of DNA fragments

DNA fragments were isolated via gel electrophoresis and recovered from the gel using either the QIAquickTM Gel Extraction Kit (QIAGEN) or the QIAEX II Gel Extraction Kit (QIAGEN) according to the manufacturers instructions.

2.2.5.8 Southern analysis of genomic DNA

2.2.5.8.1 Transfer of DNA to the nylon membrane

Southern analysis was based on the method described by Southern (1975). DNA to be analyzed was separated by gel electrophoresis as described in section 2.2.5.8, except the agarose gel contained no EtBr as this can cause uneven background problems. The gel was then stained by incubation in 1xTAE containing 0.5µl/ml of 1mg/ml EtBr for 30min. The gel was then denatured by a further 30 min incubation in 0.4M NaOH. The apparatus for DNA transfer was assembled as described by Southern (1975), with 0.4M NaOH replacing 20xSSC as the transfer buffer. The filter used for transfer was Hybond-N^+ nylon membrane (Life Science, Leicestershire, UK). The blot was left for 16h to allow DNA to transfer to the membrane filter, which was rinsed in 2x SSC (0.3M NaCl, 30mM sodium citrate pH 7.0). Filters were aired for 10min until only moist, then covered in saran wrap and stored at -20°C. There was no need to crosslink the DNA to the filter due to the alkaline transfer method.

2.2.5.8.2 Preparation of DIG labeled probes

Probes were labeled with digoxigen-dUTP (DIG-UTP), alkali labile, using probe specific primers (Roche, East Sussex, UK). Reactions were carried out in a 100µl volume containing; 11x PCR buffer without nucleotides, 0.2mM dATP, 0.2mM dCTP, 0.2mM dGTP, 0.04 mM dTTP, 2.5u Taq Polymerase and ddH_20. 3 separate reactions were undertaken for each probe to allow analysis and maximization of the incorporation of the label. Either 0/4µM/8µM of DIG-UTP was added to each tube. 5 pmol of each probe specific primer was added after an initial incubation at 94°C for 3 min. This was followed by a standard PCR reaction as described in Section 2.2.5.4.

10µl of each PCR reaction was run on a 1% agarose gel to analyze the incorporation of the DIG-UTP. DIG-UTP is significantly larger than dUTP and its incorporation inhibits the travel of the fragment through the agarose, allowing estimation of label incorporation. If the 8µM reaction was successful this was used preferentially, due to a higher level of label incorporation.

2.2.5.8.3 Hybridization of nucleic acids on membrane with probe

Based on methods in Sambrook et al., (1989). All filters were pre-hybridized at 65°C in 50ml/tube of hybridization buffer (5x SSC, 0.1% N-lauroylsarcosine, 0.5% SDS, 1% blocking reagent (Boehringer Mannheim, East Sussex,UK) and 100µg/ml of boiled sheared herring sperm DNA, for at least 2 h. Followed by an incubation of 16-20hr in fresh hybridization buffer containing the denatured DIG-UTP labeled probe. The filters were then washed in the following solutions: 2 x 5min in wash 1 (2xSSC, 0.1% SDS), 2 x 5min in wash 2 (0.1% SSC, 0.1% SDS), 1 x 1min in buffer 1 (100mM Tris-HCl pH 7.5, 150mM NaCl) and 1 x 30min in buffer 2 (buffer 1 with 1% marvel). All washes were carried out at room temperature.

The filters were then incubated with the Anti Digoxigenin antibody for 45 min according to the manufacturers instructions (Boheringer Manheim) and then washed in the following solutions: 2 x 15 min in buffer1, 4 x 5min in buffer2, 4 x 5min in buffer 1 and 1 x 5 min in buffer 3 (50mM Na_2CO_3, 1mM MgCl added to 50mM

$NaHCO_3$, 1mM MgCl until solution reaches pH 9.5). The filters were then incubated with the CPD Star substrate (Promega, Southampton, UK) according to the manufacturers instructions and exposed to X-Ray film.

2.2.6 Transformation of bacteria

2.2.6.1 Preparation of chemically competent *E.coli*

Chemically competent *XL1* blue *E.coli* were prepared based on a method by Sambrook et al., (1989). A single colony was used to inoculate 5ml of 2xTYwith tetracyclin added to 10μg/ml, and shaken at 37°C, 200rpm overnight. 50ml of 2xTY was inoculated with 0.5ml of this culture and incubated at 37°C, 200rpm until an OD_{600}= 0.6 was reached. The cells were pelleted by centrifugation at 1000Xg at 4°C for 10 min. The supernatant was carefully removed and the cell pellet resuspended in 0.5vol of ice-cold 50mM $CaCl_2$ and incubated on ice for 1 hour. After centrifugation at 1000g (3000rpm) for 10min at 4°C the pellet was resuspended in 0.1vol of ice cold (20% glycerol, 50mM $CaCl_2$) and 100μl aliquots flash frozen in liquid N_2. The cells were then stored at -80°C. Subcloning efficiency TM *DH5α* competent cells (GibcoBRL, Paisley, UK) were also used according to the manufacturers instruction.

2.2.6.2 Preparation of electrocompetent *A.tumefaciens*

Electrocompetent *A.tumefaciens* were prepared and transformed using a protocol adapted from Wen-Jen and Forbe (1989). The bacteria were grown at 28°C for 24 to 30h to an OD at 600nm of 0.5-0.7. The cells were harvested by centrifugation in at 3000rpm for 10 min. The pellet was successively washed in 1, 0.5, 0.1, 0.02 culture volumes of ice cold 10% (v/v) glycerol, and finally resuspended in 0.01 culture volumes. Aliquots of 40μl were snap frozen in liquid N_2 and stored at -80°C until required.

2.2.6.3 Transformation of *E.coli* XL1 blue

Based on a method by Sambrook et al., (1989). 100µl aliquots of chemically competent cells (section 2.2.6.1) were thawed on ice and 10µl of 10x TCM (100mM Tris-HCL pH7.5, 100mM $CaCl_2$, 100mM $MgCl_2$) added. 5µl (50ng) of ligated DNA was added, mixed gently and placed back on ice for 30 min. The cells were heat shocked at 42°C for 45 sec and placed immediately back on ice for 5 min. 1ml of 2xTY media was added and the tube incubated at 37°C, 200rpm for 30min. 100µl and 500µl fractions were plated on 2xTY agar plates and incubated at 37°C overnight.

2.2.6.4 Electroporation of *A.tumefaciens*

Aliquots of 40µl of electrocompetent cells were thawed on ice before transfer to a pre-cooled 0.2cm electroporation cuvette (Bio-Rad). 1µl of the required plasmid DNA was added and mixed with the cells on ice. A Gene PulsarTM and pulse controller unit (Bio Rad) were to transform the cells, using a field strength of 12.5kV/cm, a capacitance of 25 µF and resistance of 400 ohms. Immediately after electroporation 1ml of 2xTY media was added and mixed gently with the cells. Aliquots of 100µl and 800µl were plated on 2xTY agar containing the required antibiotics for selection and incubated at 28°C for 24h.

2.2.7 Plant transformation

2.2.7.1 Preparation of *A.thaliana* plants for infiltration

The *A.thaliana* ecotype Col-0 was used for all infiltration experiments. Seeds were sown evenly on to soil in either square $9cm^2$ pots or larger circular pots of diameter $14cm^2$. 3 to 4 seeds were used for the smaller pots and approximately 6 seeds for the larger pots. After an initial stratification period of 4 days at 4°C the pots were covered and moved to a glass house. The covers were removed after 2 weeks and the plants grown for a further 2 to 3 weeks until the main bolt had reached 10cm in length. The main bolt was then removed to promote the growth of multiple secondary bolts. The plants were then grown for a further 3 to 7 days prior to use.

2.2.7.2 Preparation of *A.tumefaciens* culture for transformation

2 days prior to transformation 10 ml of 2xTY media containing spectomycin at 100μg/ml was inoculated with a single transformed *A.tumefaciens* colony carrying the required construct. The culture was grown overnight in a shaking incubator at 28°C and 200rpm. This culture was then used to inoculate a further 400mls of 2xTY containing spectomycin at 100μg/ml, which was also grown overnight at 28°C and 200rpm. 400mls of culture was used for 4x 9cm square pots and 2x 14cm circular pots.

2.2.7.3 Floral dipping

A.tumefaciens were harvested by centrifugation at 3000rpm for 10 min at room temp. The resulting pellet was resuspended in 400ml of a fresh 5% sucrose solution by gentle shaking and Silwett L-77 (LEHLE SEEDS) added to a final concentration of 0.025%. The *A.tumefaciens* solution was then place in a plastic container, 11cm by 18cm and 7cm deep. The floral parts of the plants were then dipped in to the solution for 10sec with gentle agitation. Plants were then watered, covered overnight to create a humid environment then transferred to a glass house.

2.2.7.4 Harvesting of seeds

Mature seeds were collected from dipped plants over a period 3 to 5 weeks after they had been dipped. Watering of the plants was stopped approximately 3 weeks after dipping to aid dessication. Collected seeds were stored in paper envelopes until required after which they were separated from the majority of other plant material using a 500nm diameter sieve.

2.2.7.5 Selection for transformants

1g of seed (approximately 50 000 seed) were weighed into a 50ml tube. The seed were then surface sterilized for 5 min in 25ml of 70% ethanol, 5 min in 25ml of 50% bleach with Tween 20 (Sigma) added to a final volume of 0.05% and washed 6 times

in ddH_20. Seeds were allowed to settle between each step and the wash removed by decanting.

The last wash was replaced with 45ml of sterile 0.8% agarose kept at 45°C. 9ml aliquots were pipetted onto 140mm petri dishes containing plant growth media with kanamycin added to a final concentration of 50μg/ml to select for resistant seedlings. Plates were sealed with parafilm and after an initial stratification period of 4°C for 5 days were transferred to a growth room at 25±2°C, 16 h light.

After 2 weeks seedlings resistant to kanamycin were identified with ease. Resistant seedlings were of taller in height and with green leaves and long roots. Untransformed plants were pale, stunted in height and had limited root growth. Potential transformants were carefully removed from the agarose, briefly washed in tap water and transferred to soil and grown as described in Section 2.2.1. Confirmation of the incorporation of the transgene was carried out by PCR on DNA from the leaf of the primary transformant.

2.2.7.6 Selection for transformants with a single locus transgene insertion

Primary (T1) transformants selected in 2.2.7.5 were allowed to self-fertilize to give secondary (T2) transformant seed. A sample of approximately 100 T2 seed was screened for kanamycin resistance as described in section 2.2.7.5. When a ratio of approximately 3:1 resistant to susceptible seedlings was obtained this indicated that the line carried a transgene insertion at a single locus. These lines were used for further study.

2.2.8 Histochemical localization of GUS activity in developing seeds and ovules

Seeds were processed using a protocol adapted from Sessions et al., (1999). Seeds and ovules were dissected from pods and gynociea respectively, in a drop of staining solution (50mM $NaPO_4$ buffer pH 7.0, 10mM EDTA, 2mM $K_4Fe(CN)_6.3H2O$, 2mM $K_3Fe(CN)_6$, 0.1% TritonX-100, 1mg/ml X-GLUC and chloramphenicol added to 80μg/ml) on a microscope slide. The tissue was then carefully transferred to a well of a square petri dish and staining solution added to cover the sample. The plates were sealed with parafilm, covered in foil and incubated overnight at 37°C. Seeds and ovules were transferred to a microscope slide and the remaining staining solution removed with tissue. The material was cleared in Chloral hydrate solution (8 parts Chloral hydrate, 1part glycerol, 3 parts H_20) and visualized with a Nikon E800 microscope equipped with Normaski optics.

2.2.9 Image capture and processing

Mature seed photographs were originally obtained using an Olympus camera and Kodak film and processed at Redcliffe Laboratories (Bristol, UK). Later pictures were captured with a Nikon digital camera (Nikon, Tokyo, Japan). Images of histochemically stained seed, ovules, pollen and flowers were obtained with a Spot RT color camera (Diagnostic instruments inc, Michigan, USA) using the Spot advanced program. Images of the stained flowers were also captured with the digital Nikon camera. All images were processed with Adobe Photoshop version 4.0.1 or 5.0.

Chapter 3

The role of DNA methylation in the parent-of-origin effects on seed development in *A.thaliana*

3.1 Introduction

As described in Section 1.6 the aim of the work reported in this Chapter was to determine whether DNA methylation has a role to play in the parent-of-origin effects on seed development, and by inference genomic imprinting, in *A.thaliana*. The role of DNA methylation in mammalian imprinting was first shown in mice homozygous for a null mutation in the *Dnmt1* methyltransferase gene (Li et al., 1992 ; 1993). The mice not only showed a dramatic reduction in genome wide methylation, but also the loss of monoallelic expression of various imprinted genes. We therefore proposed that if methylation plays a similar role in the parent-of-origin effects, and by inference imprinting, in plants, the attenuation of imprinting-associated methylation can be predicted to have certain effects on imprinted plant gene expression. Namely that the prevention of imprinting- specific methylation will result in the loss of monoallelic expression of hypomethylated imprinted genes in the endosperm. These predictions are illustrated in Figure 3.1. In the wild type situation, imprinted genes are differentially methylated in the gametes and are subsequently expressed or silenced in the endosperm, depending on their parent-of-origin. If this imprinting-associated methylation is prevented, the scenario illustrated in Figure 3.1B, gametes are produced that contain alleles that are normally subject to imprinting, that after fertilization are no longer silenced in the developing endosperm.

In contrast to the situation in mammals, where a significant number of imprinted genes have been identified, at the time the experiments outlined here were designed only one gene (*MEA*) was suspected to be imprinted in *A.thaliana* (Grossniklaus et al., 1998). Therefore testing directly the loss of monoallelic imprinted gene expression was not feasible. However, we did have a method by which we could phenotypically measure an

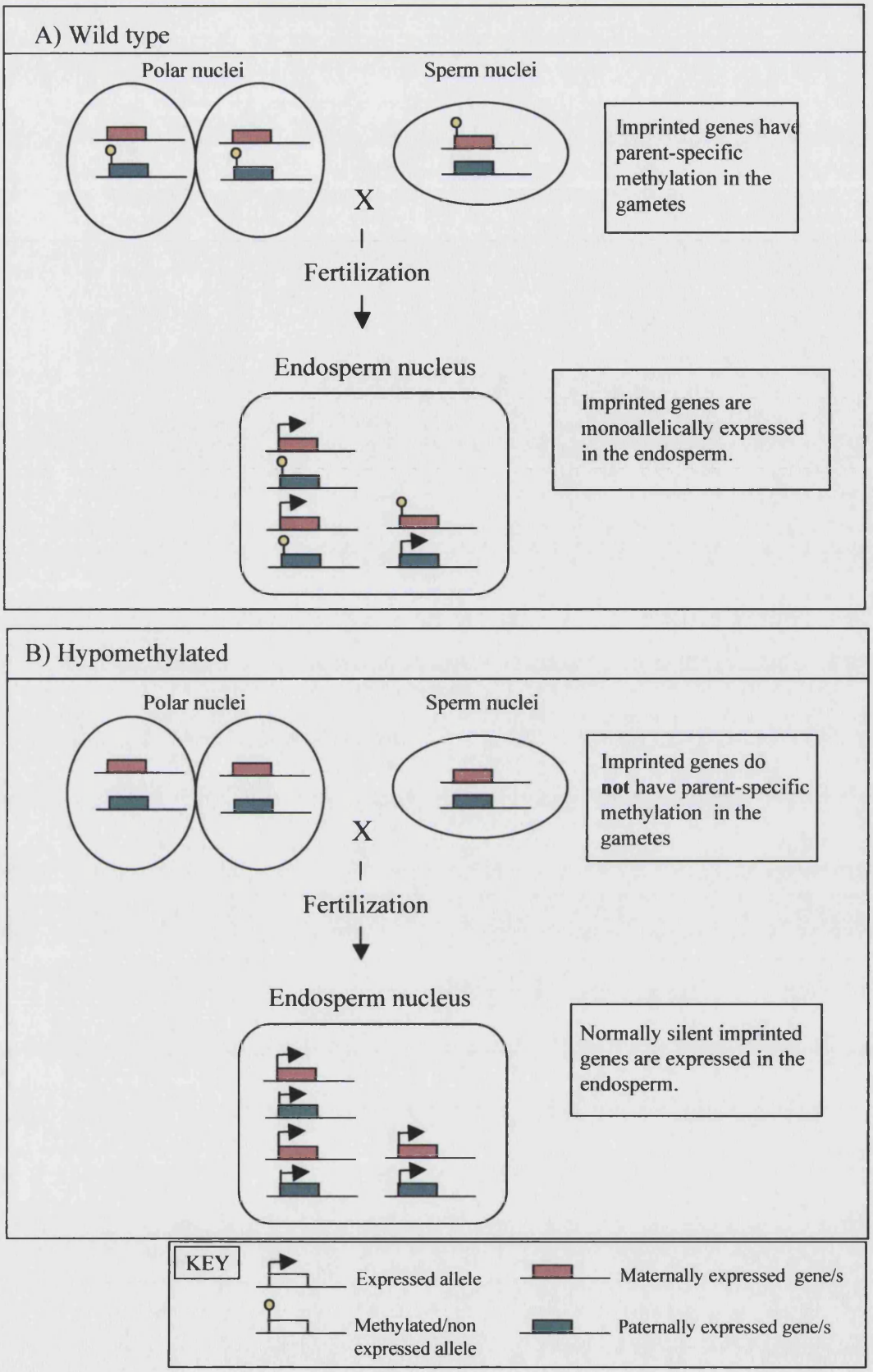


Figure 3.1
Predicted status of imprinted alleles if methylation has a role in imprinting in *A.thaliana* in (A) wild-type plants and (B) in hypomethylated plants.

imbalance of genomes, and by inference an imbalance of imprinted gene expression, in the endosperm of seed. If overall, additional maternal genomes are supplied to the endosperm (for example in a [4x x 2x] cross), the resulting seed have an under developed endosperm and a small mature seed size (Scott et al., 1998). Conversely, if additional paternal genomes are added (for example in a [2x x 4x] cross) the seed have an over developed endosperm and a large mature seed size.

Thus, the prediction that methylation is important in plant imprinting can be tested indirectly by crossing hypomethylated plants with wild type plants and measuring the effect on endosperm and seed development (Figure 3.2). For example, a hypomethylated paternal plant would be predicted to contribute extra active (endosperm-inhibiting) alleles to the endosperm that are usually only expressed from the maternally derived genome. In crosses with a wild type maternal plant this would result in a maternal excess imbalance of imprinted genes in the endosperm, giving a seed with an under proliferated endosperm and a small mass. In contrast, a hypomethylated maternal parent would be predicted to contribute extra active (endosperm-promoting) paternal specific alleles to the endosperm. In crosses with a wild type paternal parent this would result in a paternal excess imbalance of imprinted gene expression in the endosperm, leading to a seed with an over proliferated endosperm and a large mature mass.

Therefore we proposed to carry out reciprocal crosses between hypomethylated plants and wild type plants and analyse the resulting seed for their endosperm and mature seed phenotype. In an ideal situation the hypomethylated plants used in these experiments should contain a null gene (for example by a T-DNA insertion in the gene) for the methyltransferase involved in imprinting. However, there are a number of different putative methyltransferase genes in *A.thaliana* and therefore a systematic approach encompassing all the predicted methyltransferases was proposed. The screen for these T-DNA lines is outlined in Chapter 4.

At the beginning of the experiments no such lines were available. Instead, a transgenic line carrying a *METHYLTRANSFERASE* antisense (*MET1a/s*) transgene driven by the

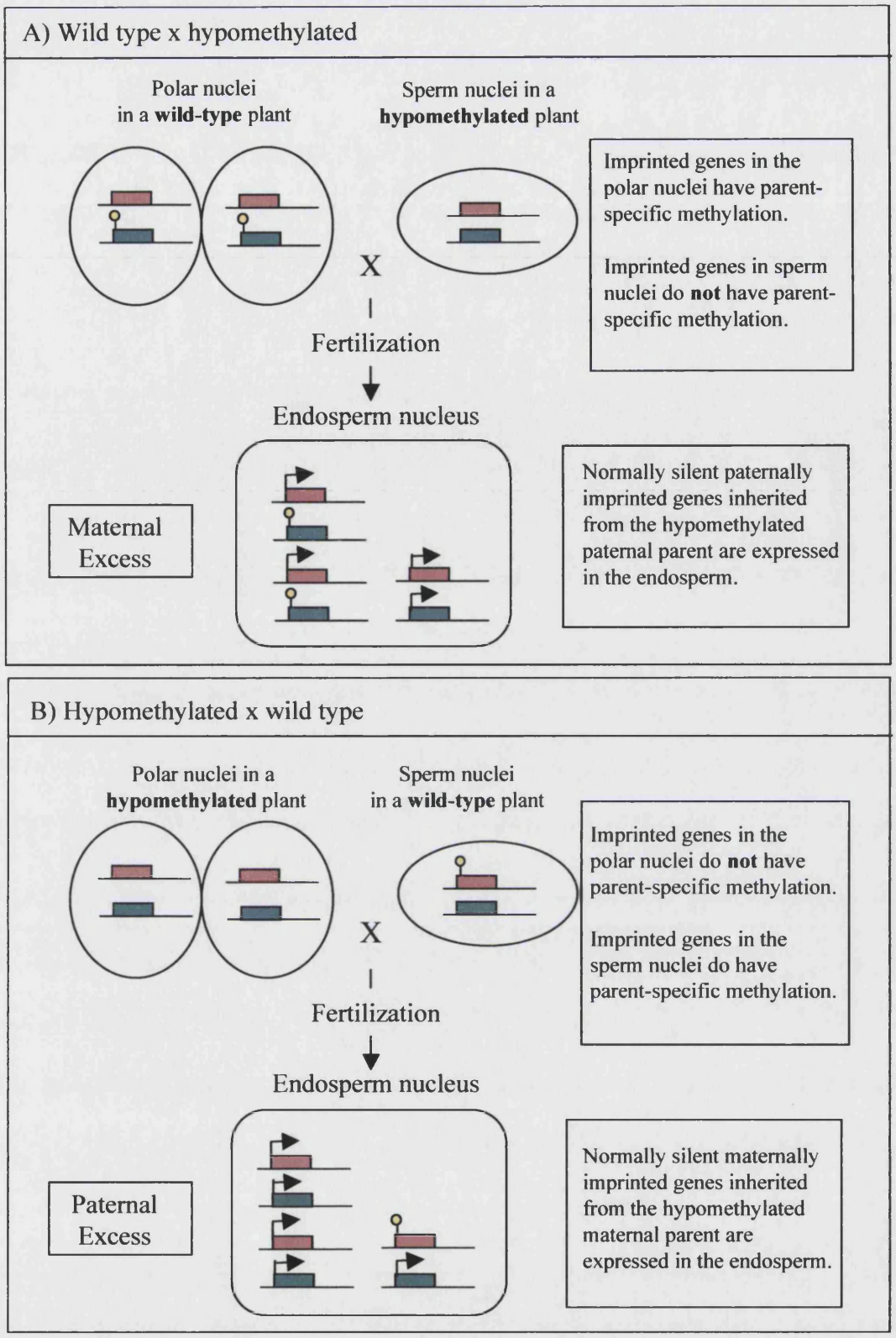


Figure 3.2
Predicted status of imprinted genes in the progeny from reciprocal crosses between hypomethylated and wild type plants, if methylation has a role in the parent-of-origin effects. In a wild type x hypomethylated cross (A) we predicted a maternal excess phenotype in the seed as the hypomethylated paternal parent contributes extra active maternal alleles. In a hypomethylated x wild type cross (B) we predicted a paternal excess phenotype in the seed as the hypomethylated parent contributes extra active paternal alleles. For the key see Figure 3.1.

35SCaMV promoter was obtained from Jean Finnegan (Finnegan et al., 1996; Genger et al., 1999). These lines had genomic methylation levels reduced to 13% of the wild type level. *MET1* was the only *A.thaliana* gene at the time to show potential methyltransferase activity and was predicted, like DNMT1 in mammals, to be the main maintenance methyltransferase (Finnegan et al., 1993; Finnegan et al., 1996; Ronemus et al., 1996). Therefore it was hoped that the dramatic reduction in *MET1* activity would have a similar effect on imprinting as knocking out *Dnmt1* activity in mice. Hence these *MET1a/s* plants were used as the hypomethylated plants in the experiment. However, this strategy contained a number of potential problems.

1) If methylation does have a role in the parent-of-origin effects in *A.thaliana* it may be established or propagated by a methyltransferase other than the *MET1* encoded enzyme. This problem is addressed in Chapter 4.
2) If MET1 does play a role it may not be the only or primary DNA methyltransferase required for the parent-of-origin associated methylation. In this case the predicted endosperm and mature seed phenotypes in the progeny from the crosses may be too subtle to be observed clearly. To tackle this we proposed to conduct reciprocal crosses between *MET1a/s* and 4x wild type plants. It was predicted that any further imbalance of imprinted gene expression superimposed on the parental excess of genomes in the seed from either a [2x X 4x] or [4x X 2x] cross created by using a *MET1a/s* plant as the 2x parent would result in a more extreme endosperm and seed phenotype. As seed from crosses between 2x and 6x plants invariably abort (Scott et al., 1998), we predicted that crosses between *MET1a/s* and 4x plants would give a high rate of aborted seed. The predicted phenotypes of these crosses are illustrated in Table 3.1.
3) Unlike a T-DNA insertion in a gene that can result in a null allele, the reduction of MET1 activity in *MET1a/s* plants relies on the expression of the antisense *MET1* RNA from the 35CaMV promoter. If MET1 is important in plant imprinting, but the 35S CaMV promoter is not active at the critical developmental time points, then the MET1 enzyme could still be active, and the predicted parent- of-origin effects may not be observed. Again this problem will be addressed in Chapter 4.

4) If the *MET1a/s* transgene is active in the endosperm this could prevent MET1 associated methylation in the endosperm. This could result in MET1 catalysed imprinting-associated methylation being lost (as the methylation of the DNA is not replicated through cell division) from chromosomes inherited from the wild type parent. This would diminish the predicted parent-of-origin effects illustrated in Figure 3.2, Table 3.1. Therefore, the absence of the parent-of-origin effects in reciprocal crosses involving one demethylated plant could reflect loss of imprints from the chromosomes inherited from the normally methylated parent. We proposed to tackle this problem by repeating the crosses between hypomethylated plants and wild type plants using a hypomethylated plant that was hemizygous for the *MET1a/s* transgene. In these crosses only half of the progeny inherited the transgene allowing the examination of the effect of the transgene on endosperm and seed development after fertilization.

Therefore the ultimate aim of this chapter was to determine if DNA methylation plays a role to in the parent-of-origin effects, and by inference imprinting, in the plant species *A.thaliana*. For this purpose we proposed to carry out a series of reciprocal crosses between hypomethylated plants (carrying the *MET1a/s* transgene) and wild type plants and to study the resulting seed for parent-of-origin effects on endosperm development and mature seed size.

Table 3.1

The predicted endosperm and mature seed phenotypes of seed resulting from crosses between *MET1a/s* and wild type 2x and 4x plants.

Cross	Status of imprinting in the endosperm	Predicted imbalance of imprinted genes in the endosperm	Predicted endosperm phenotype	Predicted mature seed size compared to [2x X 2x] seed	Predicted germination rate %
2x X 2x	Maternal and paternal imprinting	Balanced	Wild type		100
MET1a/s X *MET1a/s*	No maternal or paternal imprinting	Extra active endosperm promoting and endosperm limiting alleles	Proliferation of the endosperm comparable to that in a [2x X 2x] seed.	Larger [1]	100
MET1 a/s X 2x	No maternal imprinting Paternal imprinting	Extra active endosperm promoting alleles	Greater proliferation of the endosperm compared to a [2x X 2x] seed.	Larger	100
MET1 a/s X 4x	No maternal imprinting Paternal imprinting Extra paternal genomes (from 4x plant)	Extra active endosperm promoting alleles. Extra paternal genomes	Greater proliferation of the endosperm compared to a [2x X 4x] seed.	Smaller (aborted)	0
2x X *MET1a/s*	Maternal imprinting No paternal imprinting	Extra active endosperm limiting genes	Less proliferation of the endosperm compared to a [2x X 2x] seed	Smaller	100
4x X *MET1a/s*	Maternal imprinting No paternal imprinting Extra maternal genomes (from 4x plant)	Extra active endosperm limiting genes Extra maternal genomes	Less proliferation of the endosperm compared to a [4x X 2x] seed	Smaller (aborted)	0

[1] A larger seed size is predicted as the maternal parent contributes twice as much genetic material (and therefore more extra active endosperm-promoting alleles) than the paternal parent, to the endosperm. Overall this should give a paternal excess imbalance in the endosperm resulting in a larger seed.

3.2 Results

3.2.1 The parent-of-origin effect on mature seed phenotypes in reciprocal crosses between *MET1a/s* plants and wild type 2x or 4x plants

The aim of this section of work was to determine if methylation played a part in the parent-of-origin effect on mature seed size. If crosses between *MET1a/s* and wild type plants gave the predicted reciprocal seed mass phenotypes and rates of germination (Figure 3.2; Table 3.1) this would provide evidence that methylation plays a role in the parent-of-origin effect, and by inference imprinting, in *A.thaliana.*

MET1a/s plants were crossed with wild type 2x and 4x plants and mature seed collected, weighed and subjected to a germination assay. The results are shown in Figure 3.3-3.5. For the purpose of comparison the results previously reported by Scott et al., 1998 for the seed from interploidy crosses are also shown. Seed from [4x X 2x] and [2x X 4x] crosses exhibited a high frequency of viability. [4x X 2x] seeds were lighter than [2x X 2x] seed, (15μg compared with 21μg), while [2x X 4x] seed were heavier (54μg). Seed from the extreme crosses [6x X 2x] and [2x X 6x] were shrivelled and inviable. Seed from crosses with *MET1a/s* and wild type plants showed a similar parent-of-origin effect on mature seed phenotype.

[2x X *MET1a/s*] seed were lighter than [*MET1a/s* X *MET1a/s*] (10μg compared with 14μg), while the reciprocal cross [*MET1a/s* X 2x] gave heavier seed (33μg). In all three crosses most seed was viable. In contrast, in the [4x X *MET1a/s*] and [*MET1a/s* X 4x] crosses viability dropped off sharply, but was not reduced to 0 as for [2x X 6x] and [6x X 2x] crosses. The mean seed weight was also higher for the seed from *MET1a/s* crosses with 4x plants, compared to seed from the wild type crosses between 2x and 6x plants. These results are consistent with the model of MET1 DNA methylation having a role in the parent-of-origin effects (Figure 3.2, Table 3.1).

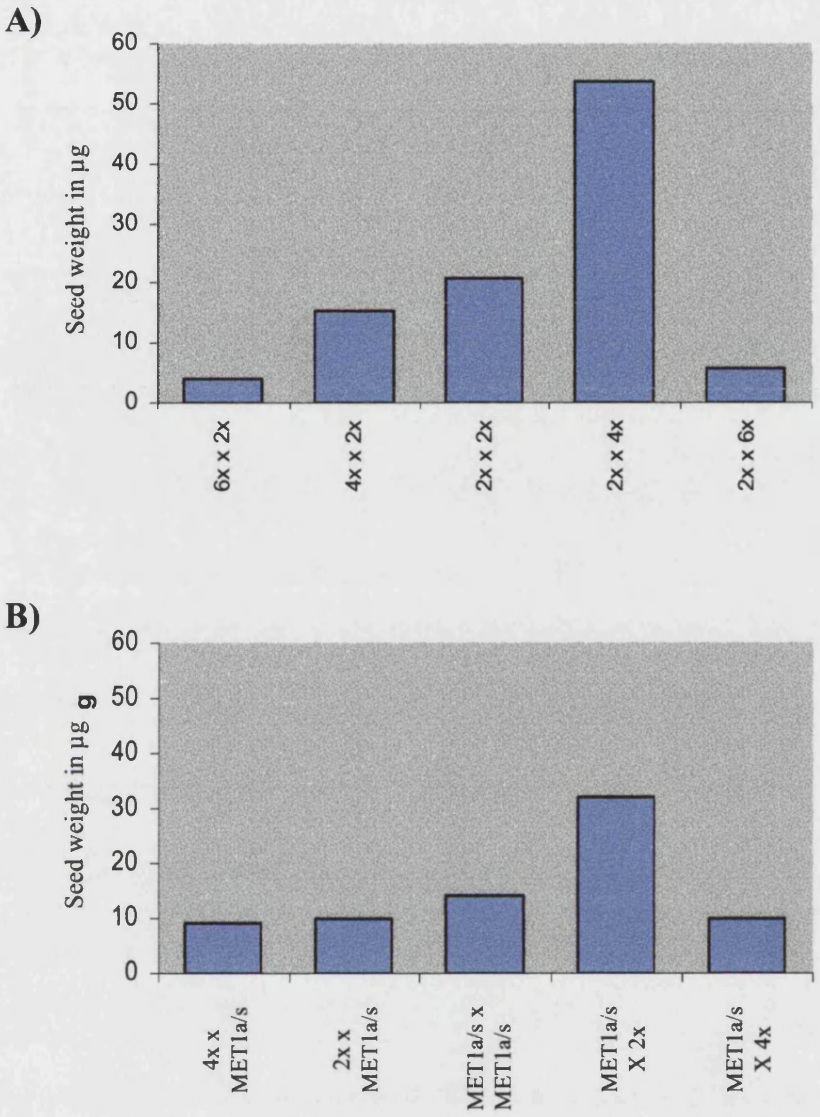


Figure 3.3
Mean weights of seed from interploidy crosses (A) and crosses using *MET1a/s* plants (B).

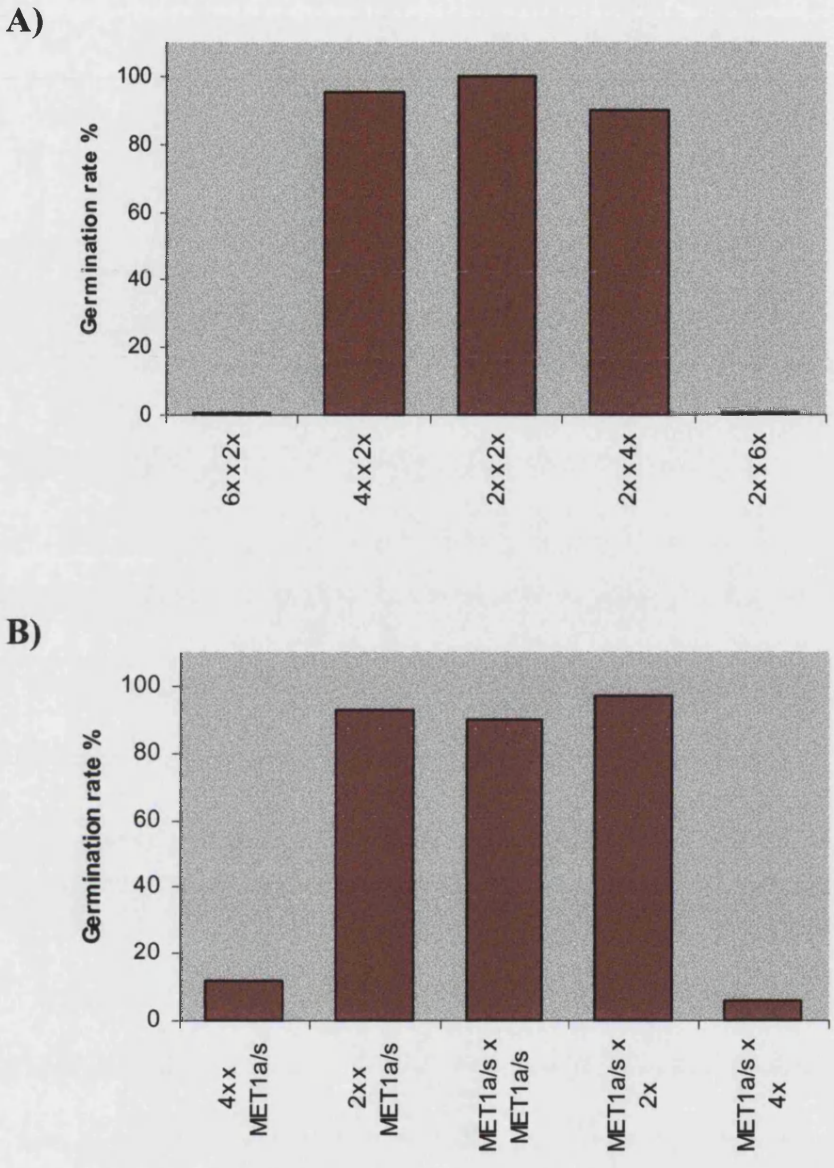


Figure 3.4
Mean germination frequencies of seed from interploidy crosses (A) and crosses using *MET1a/s* plants (B).

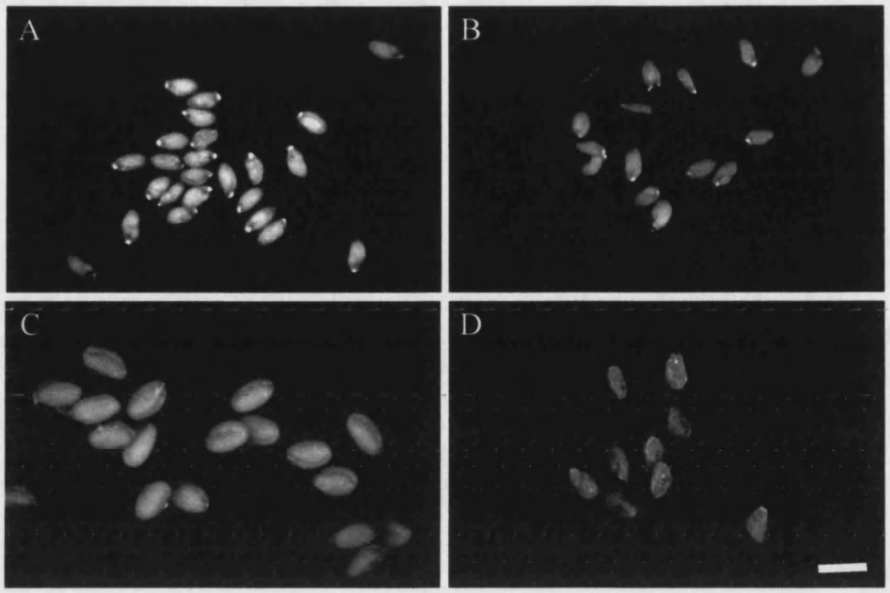


Figure 3.5

Mature seed from reciprocal interploidy crosses between 4x and wild type 2x plants (A and C) or 4x and *MET1a/s* plants (B and D). (A) [4x X 2x] seed (B) [4x X *MET1a/s*] seed (C) [2x X 4x] seed (D) [*MET1a/s* X 4x) seed. Scale bar, 1mm.

3.2.2 The parent-of-origin effect on endosperm development in reciprocal crosses between *MET1a/s* plants and wild type 2x and 4x plants

Interploidy crosses with wild type plants show parent-of-origin effects on endosperm development. If crosses between *MET1a/s* plants and wild type plants produced seed with similar parent-of-origin dependent endosperm phenotypes (Figure 3.2, Table3.1) this would provide evidence that methylation had a role in the parent-of-origin effect on endosperm development in *A.thaliana*. *MET1a/s* plants were crossed with wild type 2x and 4x plants and the seed observed for the development of the endosperm using confocal microscopy. Endosperm development was quantified in three ways:

1) Maximum size of the chalazal endosperm
2) Point of seed development (DAP) at which the PE cellularizes
3) Number of PE nuclei at the time point of PE cellularization.

Figure 3.6 shows confocal micrographs of seed from crosses with *MET1a/s* plants and show the maximum size of the CE and the point of PE cellularization for each cross. The maximum number of PE nuclei counted for each cross is shown in Figure 3.8. For the purpose of comparison results previously published in (Scott et al., 1998) for the interploidy crosses with wild type plants are shown in Figure 3.7.

Endosperm development in [*MET1a/s* X *MET1a/s*] seed was not identical to that in wild type [2x X 2x] seed. The [*MET1a/s* X *MET1a/s*] seed had a smaller CE and no chalazal nodules. However, the PE of [*MET1a/s* X *MET1a/s*] seed cellularized at a slightly later time point than in a [2x X 2x] seed (5 to 6 DAP compared to 5DAP) and produced on average more PE nuclei (598 ± 126 (mean ± s.e.m.) compared to 429 ±31).

The reciprocal crosses between *MET1a/s* and wild type 2x plants gave seed with complementary endosperm phenotypes that were comparable to those observed in seed from interploidy crosses. The cross [*MET1a/s* X 2x] gave seed with a larger endosperm than [*MET1a/s* X *MET1a/s*] seed. The CE and nodules were comparatively over grown

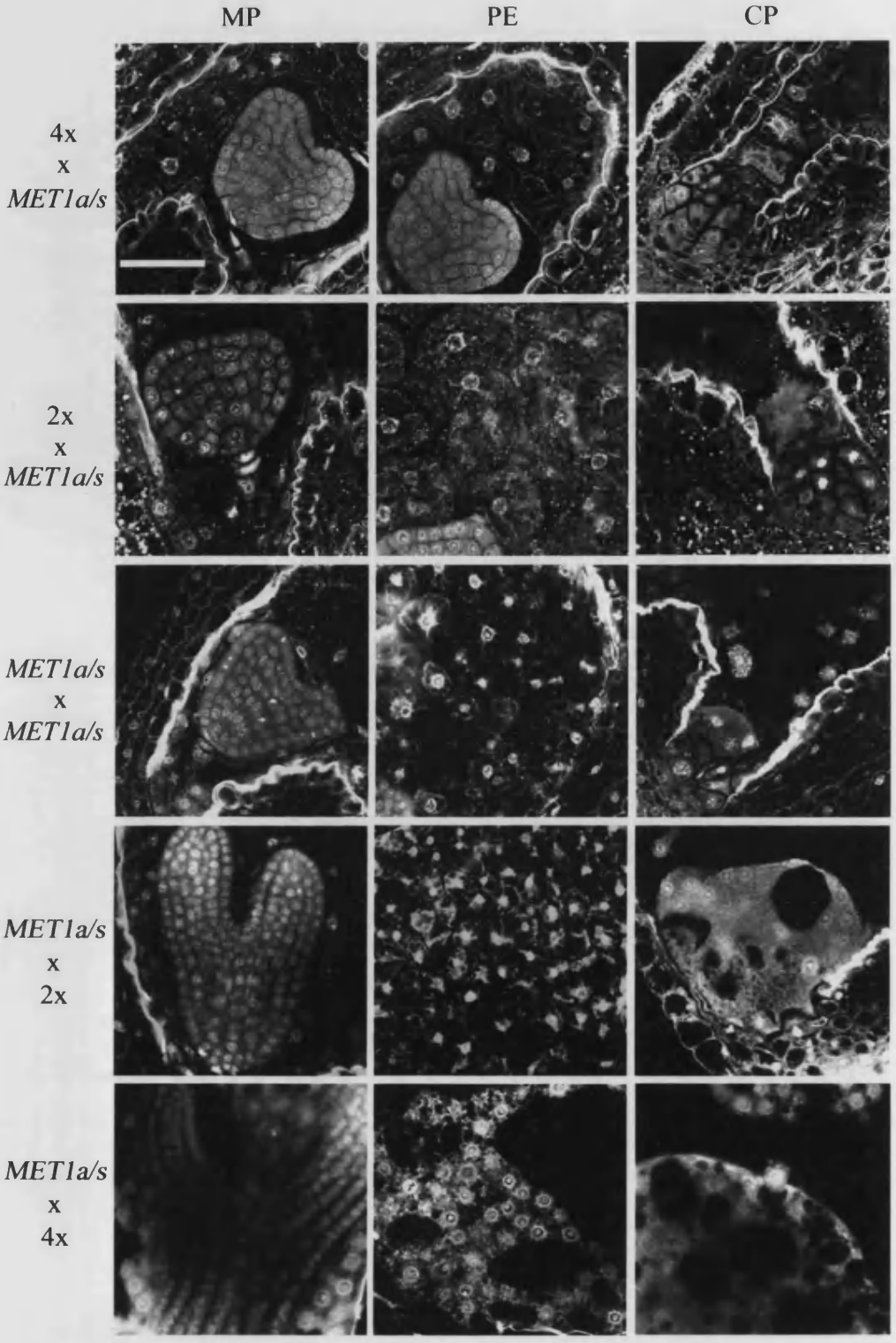


Figure 3.6
Confocal pictures of Feuglen-stained seed from crosses with *MET1a/s plants*. Images were taken at different numbers of days after pollination (DAP) but reflect typical features of the seed from each cross. (2x X *MET1a/s) and* (4x X *MET1a/s*) seeds (both 5DAP) had maternal excess phenotypes, while (*MET1a/s* X 2x) and (*MET1a/s* X 4x) seeds (both 7DAP) had phenotypes typical of paternal excess. (*MET1a/s* X *MET1a/s*) seed (6DAP) had features of both maternal and paternal excess. MP, micropylar pole; PE, central peripheral endosperm; CP, chalazal pole. Scale bar, 50um.

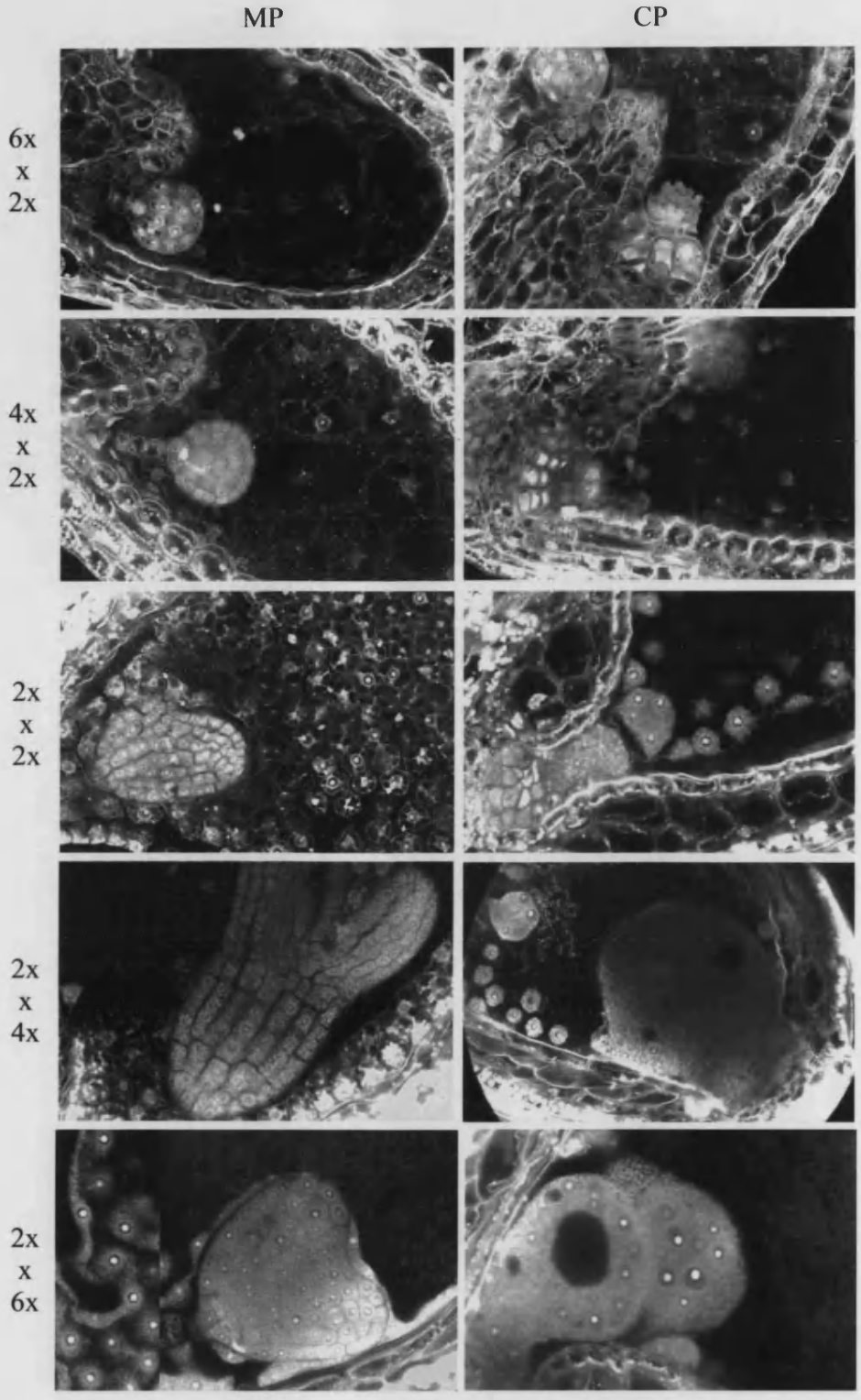


Figure 3.7

Confocal micrographs of Feulgen-stained seeds from interploidy crosses with wild-type plants. Again pictures were taken at different DAPs but reflect typical features of seeds with parental genome balance ([2x X 2x), 5DAP), maternal genomic excess ([6x X 2x], 4DAP and [4x X 2x], 5DAP) or paternal genomic excess ([2x X 6x], 6DAP and [2x X 4x], 5DAP).

and the PE cellularized relatively late (6 to 7 DAP). The PE also contained a larger number of PE nuclei (1,365±90). This over proliferation of the endosperm was similar to the development of the endosperm in seed resulting from a paternal excess interploidy cross with wild type plants (Figure 3.7-3.8, [2x X 4x]; Scott et al., 1998).

In contrast, the reciprocal cross, [2x X *MET1a/s*], produced seed with an under proliferated endosperm. The CE was small in size and no chalazal nodules were observed. The PE cellularized relatively early (3 to 4 DAP) and contained a reduced number of very large cells. These seed also produced less than half the number of PE nuclei observed in [*MET1a/s* X *MET1a/s*] seed (227 ± 17). The under proliferation of the endosperm was comparable to the development of the endosperm in seed from maternal excess interploidy crosses in wild type plants (Figure 3.7-3.8, [4x X 2x]; Scott et al., 1998).

The reciprocal crosses between *MET1a/s* and wild type 4x plants also gave seed with complementary endosperm phenotypes that are more extreme than those observed in the 2x crosses (Figure 3.6). [*MET1a/s* X 4x] seed had a highly over proliferated endosperm. The CE and nodules were extremely over grown and contained large and numerous vaculote regions. The PE showed no indications of cellularization at 10DAP (the last time point the seeds can be viewed with the confocal microscope). On average [*MET1a/s* X 4x] seed have slightly fewer PE nuclei than [*MET1a/s* X 2x] plants. The drastic over proliferation of the CE endosperm and failure of the PE to cellularize (at least before 10DAP) are phenotypes previously observed in seed from extreme paternal excess interploidy crosses (Figure 3.7-3.8, [2x x 6x]; Scott et al., 1998).

In contrast, the [4x X *MET1a/s*] cross produced small seed with a highly under proliferated endosperm. The CE was small or absent and no nodules were observed. The example in Figure 3.6 shows the PE cellularized down towards the base of the chalazal pole, with no development of the CE. The PE was also extremely under proliferated, and cellularized early at 2 to 3 DAP. The seed also contained very few PE nuclei (97 ±10) (Figure 3.8). A similar dramatic reduction in the development of the endosperm had been

previously reported for seed with extreme maternal excess (Figure 3.7-3.8, [6x X 2x]; Scott et al., 1998).

In summary, a parent-of-origin effect on endosperm development and mature seed phenotype was observed in seed from crosses between *MET1a/s* and wild type 2x and 4x plants, which was consistent with the proposed model of DNA methylation having a role in the parent-of-origin effects (Figure 3.2, Table 3.1).

3.2.3 Crosses using *MET1a/s* hemizygote hypomethylated plants to determine the post-fertilization effect of the transgene on endosperm and seed development

A concern at the beginning of the experiments outlined in this Chapter was the possibility that activity of the *MET1a/s* transgene in the endosperm could result in the loss of methylation from chromosomes inherited from a wild type parent. This would diminish the predicted parent-of-origin effects on endosperm development and mature seed size in the progeny from crosses between *MET1a/s* and wild type plants. We proposed to study the post-fertilization effect of the *MET1a/s* transgene on endosperm and seed development by repeating the crosses between hypomethylated and wild type plants with hypomethylated plants that contain only one copy of the *MET1a/s* transgene. In these crosses, only half of the progeny inherit the transgene (Figure 3.9). This could result in two different scenarios depending on the activity of the *MET1a/s* transgene.

If the *MET1a/s* transgene is **not active** in the endosperm, *MET1* dependent methylation will be retained on the chromosomes inherited from the wild type parent. Therefore one class of seed would be obtained. If *MET1* dependent methylation is involved in imprinting then a parent of origin dependent phenotype would also be observed.

If the *MET1a/s* transgene is **active** in the endosperm then two classes of seed will be observed if *MET1* directed methylation has a role in the parent-of-origin effects. The seed that inherit the transgene will have lost methylation from the incoming wild type chromosomes and any parent-of-origin effect on seed development will be consequently

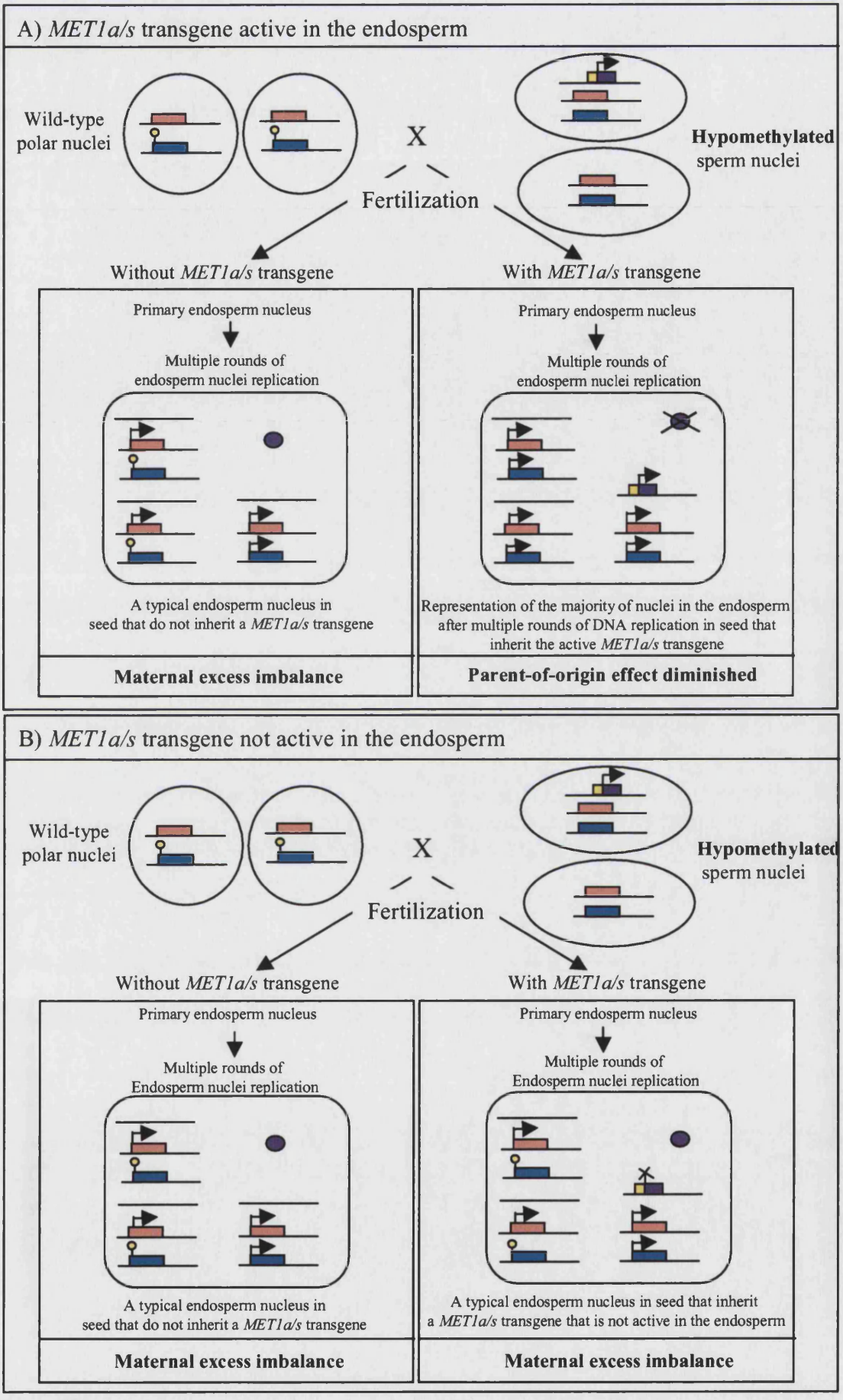


Figure 3.9

Predicted outcome of crosses between a wild-type maternal parent and a hemi*MET1a/s* paternal parent. *MET1a/s* active in the endosperm (A) two classes of seed observed. *MET1a/s* not active in the endosperm (B) one class of seed observed.

diminished. In contrast, the seed without the transgene will maintain methylation of the wild type chromosomes, resulting in a parent-of-origin effect. Therefore crosses were carried out between *MET1a/s* plants and wild type plants and the resulting seed classified with respect to endosperm development and mature seed size.

3.2.3.1 Methylation status of hemi*MET1a/s* plants

The predictions made above depend on the hemi*MET1a/s* plants producing hypomethylated imprinted alleles, despite only containing one copy of the *MET1a/s* transgene in the somatic genome. However, as with the *MET1a/s* plants it is difficult to test experimentally the hypomethylated state of gametes due to their small size and the presence of surrounding parental (somatic) tissues. It is possible though to test the methylation status of the parental plant. Hemi*MET1a/s* plants had been previously reported as having genomes that were substantially hypomethylated (Finnegan et al., 1996). To test that our hemi*MET1a/s* plants were also hypomethylated we performed analysis of the methylation status of centromeric DNA in these plants (Figure 3.10) The *Hpa II* digest of DNA from a wild type plant was inhibited by methylation of the central cytosine in the CCGG recognition sequence ($C^{m}CGG$). The *Msp I* enzyme has the same recognition site, but is not sensitive to methylation of the central cytosine, and therefore the wild type DNA cleaved. In contrast, genomic DNA from *MET1a/s* plants digested with both *HpaII* and *MspI* giving the two characteristic ladder patterns of the same intensity (Figure 3.10). This indicates that the centromeric region had significantly reduced levels of cytosine methylation in *MET1a/s* plants. The genomic DNA from the hemi*MET1a/s* plants also successfully cleaved with both the *HpaII* and *MspI* enzymes, also indicating that the centromeric region in these plants had reduced levels of cytosine methylation. However, the bands observed in the lane containing the hemi*MET1a/s* digested with the *HpaII* enzyme are consistently fainter than those observed if the DNA from the same plant was digested with the *MspI* enzyme. This indicates that the digestion of hemi*MET1a/s* plants with the methylation sensitive enzyme *HpaII* is inhibited to a greater extent than the same reaction with *MET1a/s* DNA. This suggests that cytosine methylation at the centromeric regions is reduced in comparison to wild type levels in

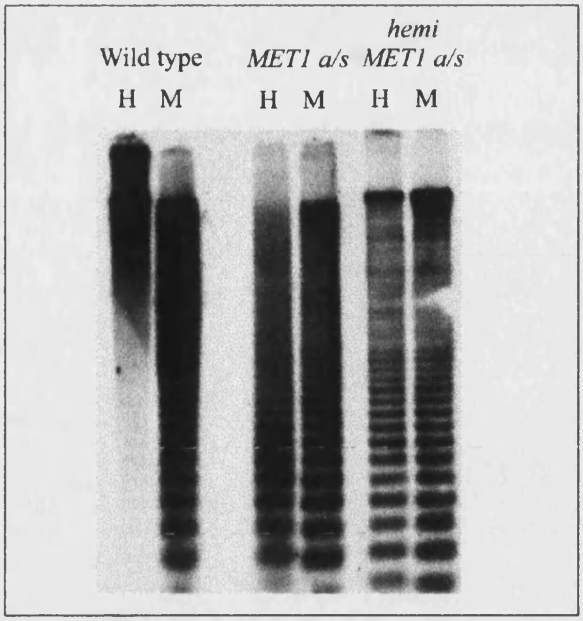


Figure 3.10

Hypomethylation of genomic DNA associated with the *MET1a/s* transgene. Southern analysis of DNA from wild-type (left), homozygous *MET1a/s* (middle) and hemi*MET1a/s* plants (right). DNA was digested with *HpaII* (H) or *MspI* (M); both cleave the sequence CCGG but the former is inhibited by cytosine methylation (McClelland *et al.*, 1994). A 180bp repeat from *A.thaliana* centromeric DNA (Martinez-Zapater *et al.*, 1986) was used as the probe.

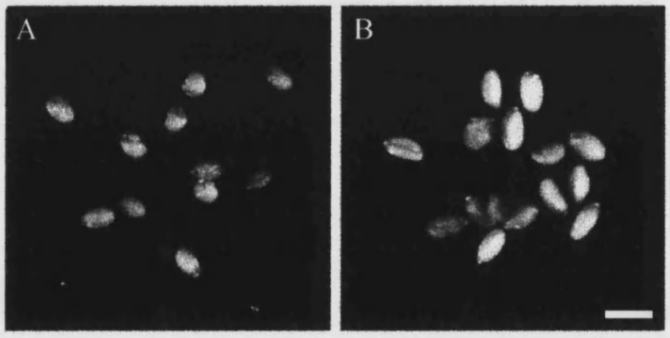


Figure 3.11

Mature seed from reciprocal crosses between hemi*MET1a/s* and wild type 2x plants. (A) seed from a [2x x hemi*MET1a/s*] cross and (B) seed from a [hemi*MET1a/s* x 4x] cross. Each cross shows a single class of seed with respect to seed size. Scale bar, 1mm.

hemi*MET1a/s* plants, but that the extent of hypomethylation is less than in *MET1a/s* plants. These results are in accordance with previous reports (Finnegan et al., 1996).

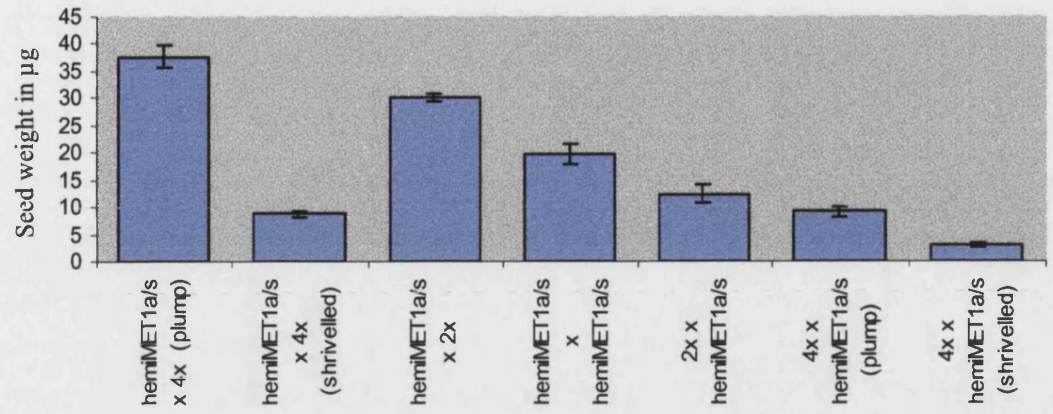
3.2.3.2 Mature seed phenotypes of progeny from reciprocal crosses between hemi*MET1a/s* plants and wild type 2x or 4x plants

The aim of this set of experiments was to analyse the post-fertilization effect of the *MET1a/s* transgene on the parent-of-origin effect on mature seed size. If the *MET1a/s* transgene is active in the developing seed we predicted that this would result in the loss of methylation from the wild type chromosomes in progeny resulting from crosses with wild type plants. If methylation has a role in the parent-of-origin effects then in this scenario crosses between hemi*MET1a/s* and wild type would plants produce two classes of seed, with the size of the seed dependent of the inheritance of the transgene. In contrast, if the *MET1a/s* transgene was not active in the endosperm we predicted that a single class of seed size would be obtained in crosses with hemi*MET1a/s* and wild type plants, with the phenotype being dependent on the methylation status of the parents. Therefore hemi*MET1a/s* plants were crossed with wild type 2x plants and the resulting seed from each cross analysed for mature mass and rate of germination. For the same reasons outlined for the *MET1a/s* crosses, hemi*MET1a/s* plants were also crossed in both reciprocal directions with wild type 4x plants.

[hemi*MET1a/s* X hemi*MET1a/s*] seed were 70% larger than [*MET1a/s* X *MET1a/s*] seed (20μg compared to 14μg). However, similar parent-of-origin effects on seed size were observed when the hemi*MET1a/s* plants were crossed with wild type plants. [hemi*MET1a/s* X 2x] seed were larger than the seed of either parental plant (30μg), while [2x X hemi*MET1a/s*] seed were smaller (12μg) (Figure 3.12). In both crosses most of the seed was viable. Most significantly analysis of the seed suggested that in each cross there was only one class of seed size (Figure 3.11).

The crosses between hemi*MET1a/s* and wild type plants gave more viable seed than the equivalent crosses with *MET1a/s* plants. On average 63% of [hemi*MET1a/s* X 4x] and

A)



B)

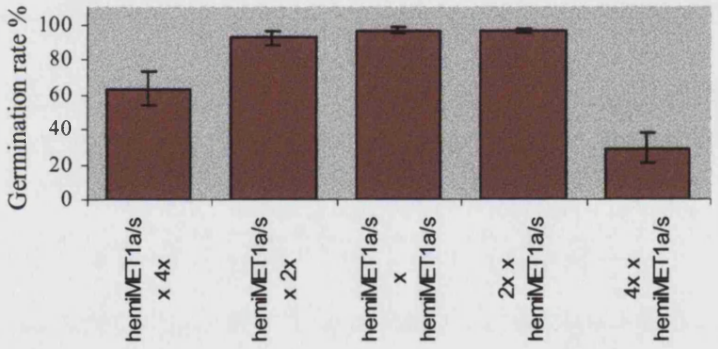


Figure 3.12

Mean weights (A) and germination frequencies (B) of seed from crosses between hemi*MET1a/s* and wild type 2x and 4x plants.

29% of [4x X hemi*MET1a/s*] were viable. However there was a large amount of variation between individual crosses. For the [hemi*MET1a/s* X 4x] cross the germination rate of seed from separate pods ranged from 6 to 100%. In the reciprocal cross, [4x X hemi*MET1a/s*], the viability of seed from individual pods ranged from 0 to 69%.

To obtain weights for these crosses the shrivelled seed were separated from the plump seed by eye and each group weighed individually. The results are shown in Figure 3.12. Plump [hemi*MET1a/s* X 4x] seed were heavier than [hemi*MET1a/s* X 2x] seed (37μg compared with 30μg), while plump [4x X hemi*MET1a/s*] seed were slightly lighter than [2x X hemi*MET1a/s*] seed (9μg compared with 12μg). In both cases the shrivelled seed was lighter than the plump seed, however the shrivelled seed from a [hemi*MET1a/s* X 4x] cross was slightly heavier than that from a [4x X hemi*MET1a/s*] cross (9ug compared to 3μg).

3.2.3.3 Segregation of the *MET1a/s* transgene in the progeny from crosses between hemi*MET1a/s* plants and wild type 2x and 4x plants

The crosses between hemi*MET1a/s* and 2x wild type plants described above indicated that the *MET1a/s* transgene was not active in the endosperm, as only one class of seed size was obtained in each reciprocal cross. However, the cross between hemi*MET1a/s* plants and 4x wild type plants gave two broad classes of seed viable (plump) and inviable (shrivelled) in each reciprocal cross. In the latter set of crosses hypomethylation is superimposed onto parental imbalance of genomes in the seed (caused by a [2x X 4x] or [4x X 2x] cross). Therefore even a small effect by the *MET1a/s* transgene after fertilization could have a drastic effect on seed development (for example reducing the seed abortion observed in cases of extreme parental excess).

According to our predictions if the *MET1a/s* transgene is active, thereby leading to the loss of methylation from wild type chromosomes and diminishing the parent-of-origin effect on seed size, then most or all of the viable seed from the crosses between hemi*MET1a/s* and 4x plants should carry the *MET1a/s* transgene. In contrast, if *MET1a/s*

is not active then half of the viable seed should contain the transgene. Although it is not possible to analyse the seed themselves for the presence of the *MET1a/s* of the transgene the resulting seedlings or plants can be tested. As the female nuclei (polar and egg nuclei) and male nuclei (sperm) of gametes each develop from a single meiotic product, if the plant contains the transgene the endosperm of the seed in which the embryonic plant developed will also have inherited the transgene.

Therefore the plants from the viable (plump) seed from each reciprocal cross between hemi*MET1a/s* and 4x wild type plants were tested for the presence of the *MET1a/s* transgene. To ensure that the transgene indeed segregates in a 1:1 ratio (and that the transgene does not confer some advantage or disadvantage on the gametes with respect to fertilization), F1 plants resulting from crosses between hemi*MET1a/s* and wild type 2x plants were also tested for the segregation of the *MET1a/s* transgene (Figure 3.13).

In a [hemi*MET1a/s* X 2x] cross approximately half of the resulting F1 plants carried the *MET1a/s* transgene (Table 3.2). Approximately half of the F1 plants from the reciprocal crosses between hemi*MET1a/s* and wild type 4x plants also contained the *MET1a/s* transgene. By inference we can conclude that 50% of the shrivelled inviable seed from these crosses would also have carried the transgene.

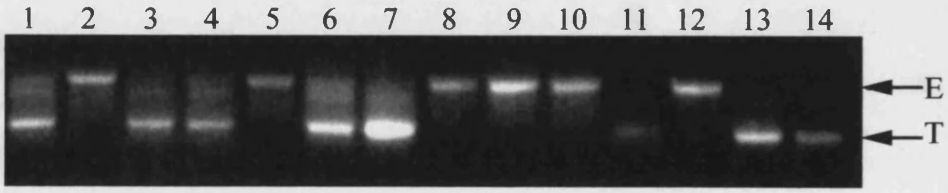


Figure 3.13

PCR products showing segregation of the *MET1a/s* transgene in progeny of a [hemi*MET1a/s* x 2x] cross (lanes 1-11); lane 12, wild type; lane 13 *MET1a/s* homozygote; lane 14, *MET1a/s* hemizygote. In our conditions the 0.7 kb transgene fragment (T) was preferentially amplified over the 1kb endogenous *MET1* gene fragment (E).

Table 3.2

Segregation of the *MET1a/s* transgene in the progeny from reciprocal crosses between hemi*MET1a/s* plants and wild type 2x and 4x plants. n is equal to the number of F1 plants tested for the presence of the transgene.

Cross	Proportion of F1 plants with the *MET1a/s* transgene %
hemi*MET1a/s* X 2x	52 n=31
2x X hemi*MET1a/s*	60 n=15
hemi*MET1a/s* X 4x[1]	50 n=12
4x X hemi*MET1a/s*[1]	45 n=11

[1]Viable seed

3.2.3.4 Parent-of-origin effect on endosperm development in the progeny from reciprocal crosses between hemi*MET1a/s* and wild type 2x plants

To analyse the action of the *MET1a/s* transgene after fertilization on the parent-of-origin effect on endosperm development crosses seed were examined at different stages from crosses between hemi*MET1a/s* and wild type 2x plants. If the *MET1a/s* transgene is active in the developing seed we predicted that this would lead to the loss of methylation from chromosomes inherited from a wild type parent. If methylation has a role in the parent-of-origin effects then we predicted that the loss of methylation from the wild type chromosomes would diminish any parent-of-origin effect on endosperm proliferation. Therefore, in this scenario, in crosses between hemi*MET1a/s* and wild type 2x plants we would observe two classes of seed with respect to endosperm development. One class of seed, not carrying the transgene, would exhibit a parent-of-origin effect on the proliferation of the endosperm (the phenotype of which would depend on the methylation status of the parents). The seed that inherited the *MET1a/s* transgene would show endosperm proliferation comparable to wild type (or more accurately to [*MET1a/s* X

MET1a/s] seed) due to loss of methylation diminishing the parent of origin effect. In contrast if the *MET1a/s* transgene was not active in the seed then we predicted that only one class of seed would be observed in these crosses with respect to endosperm proliferation.

Strikingly only one class of seed was observed in each reciprocal cross (Figure 3.14). Furthermore the seed had similar (although weaker) phenotypes to seed from crosses between wild type and *MET1a/s* plants. [2x X hemi*MET1a/s*] seed had an under developed endosperm. The CE cyst was small and chalazal nodules were rarely seen. These seed also produced fewer PE nuclei than observed in either a wild type [2x X 2x] or [*MET1a/s* X *MET1a/s*] cross. This under proliferation of the endosperm had previously been observed in both the maternal excess cross [4x X 2x] and in [2x X *MET1a/s*] seed.

In contrast, the reciprocal cross [hemi*MET1a/s* X 2x] gave seed with an over proliferated endosperm, similar to the phenotype observed in the paternal excess cross [2x X 4x] and in [*MET1a/s* X 2x] seed. The CE was over grown and a larger number of PE nuclei were produced in comparison to either a [2x X 2x] or a [*MET1a/s* X *MET1a/s*] cross.

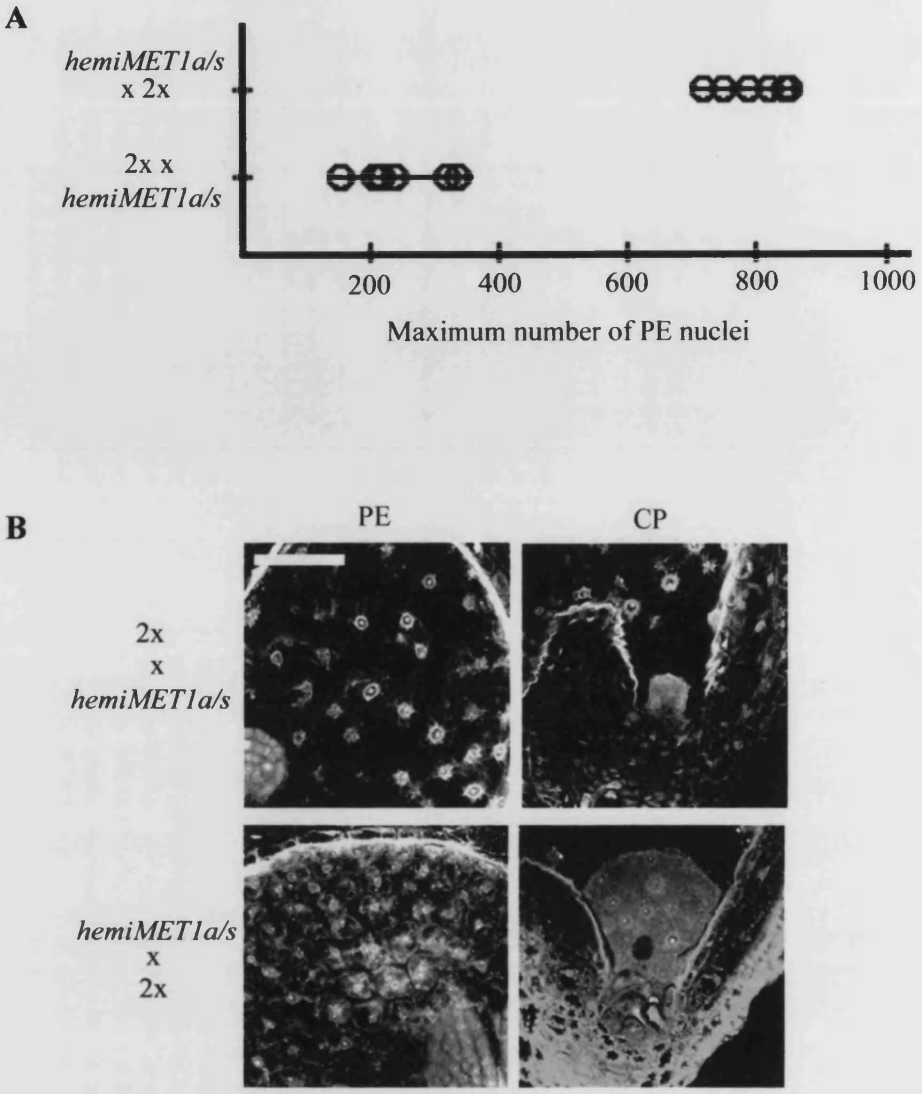


Figure 3.14

Endosperm proliferation in seed from reciprocal crosses between hemi*MET1a/s* and wild-type 2x plants (A) Number of PE nuclei (all data points shown as open circles) (B) Confocal micrographs of Feulgen-stained seeds. [2x x hemi*MET1a/s*] seeds had a maternal excess phenotype, while [hemi*MET1a/s* x 2x] seeds had a paternal excess phenotype. PE, peripheral endosperm; CP, chalazal pole. Scale bar, 50μm.

3.3 Discussion

The purpose of the work described in this Chapter was to determine if methylation, catalysed by the MET1 encoded enzyme, has a role in the parent of origin effects, and by inference imprinting, in *A.thaliana*. We predicted that if methylation has a role in plant imprinting, then preventing imprinting associated methylation would result in the expression of normally silenced imprinted genes in the endosperm. Furthermore, we proposed that if hypomethylated (*MET1a/s*) plants were crossed with wild type plants this would result in an imbalance of imprinted gene expression, comparable to the addition of extra parental genomes in the seed via interploidy crosses. Therefore *MET1a/s* plants were crossed with wild type plants and the seed observed for endosperm development, mature seed size and germination rate.

3.3.1 Crosses between *MET1a/s* plants and wild type plants produce seed with parent-of-origin effects on endosperm and seed development

Consistent with our predictions, crossing *MET1a/s* plants with wild type plants resulted in parent-of-origin effects on seed development, similar to those observed in interploidy crosses. A [*MET1a/s* X 2x] cross gave seed with an over proliferated endosperm and a large mature seed weight, which are features of a paternal excess cross (for example [2x X 4x]). This was despite the fact that both the parental plants were diploid and that the hypomethylated maternal parent, whose tissues nourished the developing seed, suffered from a number of floral and vegetative defects (Finnegan et al., 1996; Ronemus et al., 1996). The phenotypes observed fitted our prediction that if methylation by the MET1 enzyme was essential for imprinting, preventing imprinting-associated methylation of the maternal genome (with the *MET1a/s* transgene) would result in extra active endosperm-promoting alleles being contributed to the endosperm (Figure 3.2, Table 3.1), Haig and Westoby, 1989, 1991; Scott et al., 1998). In other words, the phenotype of [*MET1a/s* X 2x] seed is in accordance with a model by which the maternal *MET1a/s* parent contributes extra active alleles to the progeny that are normally only expressed if inherited from the paternal parent, phenocopying an excess of paternal genomes in the endosperm.

The reciprocal cross, [2x X *MET1a/s*], had features of maternal excess, (similar to a [4x x 2x] cross) producing small seed with an under proliferated endosperm. Again this fitted our predictions that if methylation by the MET1 enzyme is required for imprinting, preventing imprinting-associated methylation of the paternal genome would lead to extra active endosperm-inhibiting alleles being contributed to the progeny. So in crosses with wild type plants there would be an excess of alleles in the seed that are normally only expressed if maternally inherited and that this would phenocopy an excess of maternal genomes in the endosperm.

The reciprocal crosses with *MET1a/s* and 4x wild type plants also produced seed with phenotypes similar to those predicted if *MET1* is important for the parent-of-origin effects. In these crosses hypomethylation was superimposed onto parental excess. Therefore, according to our predictions the extra active imprinted alleles, contributed by the *MET1a/s* parent, pushed the seed to a more extreme parental excess seed phenotype. Indeed both a [*MET1a/s* X 4x] and a [4x X *MET1a/s*] cross produced a large number of inviable seed, in contrast to crosses between wild type 2x and 4x plants (in the ecotype C24) that produced a high proportion of viable seed (Scott et al., 1998). The high rate of seed abortion is a feature of extreme paternal excess observed in crosses between 2x and 6x plants. However, this latter set of crosses has a more drastic phenotype producing 100% aborted seed in either direction.

In addition to the low rate of germination the seed from interploidy crosses with *MET1a/s* plants showed a more extreme endosperm phenotype than the equivalent wild type crosses. [4x x *MET1a/s*] seed produced an endosperm that cellularized 2-3 DAP in contrast to the endosperm of [4x x 2x] seed that cellularized 4-5DAP (Scott et al., 1998). The reciprocal cross [*MET1a/s* x 4x], produced seed with an endosperm that failed to show any signs of cellularization 10DAP, while in [2x x 4x] seed the PE began to cellularized 6-7DAP. These more extreme endosperm phenotypes are similar to those observed in the crosses between 2x and 6x plants.

Therefore the reciprocal crosses between *MET1a/s* plants and wild type plants produced seed with parent-of-origin effects on endosperm and seed development that were similar to those observed in seed from interploidy crosses. These parent-of-origin effects were observed in the crosses with one hypomethylated plant, even if both the parents were diploid. This is evidence to support *MET1* catalyzed methylation as having a global role in the parent of origin effects, and by inference imprinting, in *A.thaliana*. However, consideration must be given to factors other than imprinting that could result in parent-of-origin effects on seed development.

3.3.2 The parent-of-origin effects on seed development could be caused by factors other than imprinting

Alternative explanations have been put forward to explain the reciprocal phenotypes of interploidy crosses and these must also be considered for the parent-of-origin effects in the *MET1a/s* crosses. The complementary phenotypes of [4x X 2x] and [2x X 4x] crosses can only be explained if the male and female gametes contribute different sets of active alleles or different sets of gene products (i.e. cytoplasmic factors) to the seed. Furthermore, the reciprocal phenotypes of [2x X *MET1a/s*] and [*MET1a/s* X 2x] seed can only be explained if uniparental hypomethylation affects sex-specific gene expression in a way that closely phenocopies interploidy crosses.

The parent-of-origin effects in interploidy crosses could be the result of a dosage imbalance of genes that are exclusively expressed in the central cell and sperm, with these products carried over into the endosperm (Birchler, 1993). With regards to the *MET1a/s* crosses, it is possible that hypomethylation allows the ectopic expression of these gametophytic genes in the wrong sex. In other words sperm specific genes are activated in the central cell and visa versa, and that this deregulated gametophytic expression alone is responsible for the phenocopy of interploidy crosses.

The central cell is a comparatively large structure that goes onto to contribute structurally to the developing seed. Therefore the 'carry over' of gene products expressed in the

central cell to the seed endosperm appears a reasonable hypothesis. However, plant sperm and generative cells (sperm precursors) are characterised by condensed chromatin, little cytoplasm and few organelles (McCormick, 1993). Furthermore very few generative cell- or sperm-specific proteins have been identified (Blomstedt et al., 1996). Thus, it is difficult to conceive how a sperm could transport sufficient gene products to have the drastic effect on endosperm proliferation and seed size observed in the paternal excess phenotype. Although possible that the parental excess phenotypes are the result of mechanism as yet unknown, a disruption of the balance of imprinted gene expression appears the strongest hypothesis at this present time.

3.3.3 Seed from a [*MET1a/s* X *MET1a/s*] cross have features of both maternal and paternal excess

We had proposed that if *MET1* was essential for plant imprinting then preventing imprinting-associated methylation in both the maternal and paternal genome would result in extra active endosperm-promoting and endosperm-inhibiting alleles being contributed to a [*MET1a/s* X *MET1a/s*] seed. Originally we had predicted that this would produce seed with an endosperm and mature seed phenotype comparable to wild type (Table 3.1). However, it should also be taken into consideration that the maternal genome contributes twice as much genetic material to the endosperm as the paternal genome (2m:1p). Therefore, on balance, we could predict that a [*MET1a/s* X *MET1a/s*] seed should have features of paternal excess, as overall the maternal genome contributes more active endosperm-promoting genes. Unexpectedly [*MET1a/s* X *MET1a/s*] seed exhibited features of both maternal and paternal excess. The seed had a small under developed CE and weighed less than a [2x X 2x] seed. These phenotypes are characteristic of a maternal excess cross. In contrast, the PE was over proliferated, producing more nuclei than a [2x X 2x] cross, a feature of paternal excess. At the present time we are not able to explain why [*MET1a/s* X *MET1a/s*] seed do not behave as predicted, although there maybe several contributing factors.

Firstly, [*MET1a/s* X *MET1a/s*] seed have two hypomethylated parents, unlike the seed from reciprocal crosses with *MET1a/s* plants and wild type plants. *MET1a/s* plants exhibit a number of developmental defects, including floral homeotic transformations caused by the misregulation of gene expression (Finnegan et al., 1996; Ronemus et al., 1996). Therefore we should carefully consider the effect of reducing methylation on factors that do not relate to imprinting. In the crosses with *MET1a/s* plants and wild type plants, genomic regions that are normally methylated are contributed to the seed by the wild type parent. This could compensate for the hypomethylated regions inherited from the *MET1a/s* parent, and thus the effect of demethylation of imprinted regions (which can not be compensated for as they are sex-specific) can be observed more clearly in these crosses. Indeed plants that are hemizygous for the *MET1a/s* transgene show far fewer developmental defects than homozygous *MET1a/s* plants, indicating that the wild type genome can reduce the effect of gene misregulation from hypomethylated alleles (data not shown). Perhaps [*MET1a/s* X *MET1a/s*] seed have the potential to develop a large CE and mature seed size but growth is inhibited by the misregulation of genes not involved in imprinting.

A second point to note is that [*MET1a/s* X *MET1a/s*] seed inherited three copies of the *MET1a/s* transgene in the endosperm. This is in contrast to [2x X *MET1a/s*] and [*MET1a/s* X 2x], which inherited one or two copies respectively. If the *MET1a/s* transgene is active in the endosperm and has a detrimental effect, this could greatly inhibit the development of a [*MET1a/s* X *MET1a/s*] seed. However, recent evidence suggests that the 35S CaMV promoter, which drives the *MET1a/s* expression, is not active in the embryo or endosperm until the embryo reaches heart to torpedo stage (Boisnard-Lorig et al., 2001). Therefore it is unlikely that the number of transgenes in the seed should have any drastic effect on seed development. The implications for the inactivity of the *MET1a/s* transgene during seed development will be discussed in greater depth later in this Chapter.

A third consideration is the possibility that preventing methylation could lead to the repression as well as the expression of imprinted genes. Indeed, experiments with murine

embryos homozygous for a null mutation in *Dnmt1,* showed the loss of expression of the imprinted loci *Igf2* and *Igf2r* (Li et al., 1993). Therefore in mammalian imprinting the default state of a gene is not necessarily a potential for expression. Hence a [*MET1a/s* X *MET1a/s*] seed could be deficient for the products of some imprinted genes, and this could limit growth. However, the *MET1a/s* plants used in these experiments have genomic methylation levels reduced to approximately 13% and are still viable (Finnegan et al., 1996). This is in contrast to murine embryos that are homozygous for the *Dnmt1* mutation, which retain 30% of the normal genome methylation levels yet abort by midgestation (Li et al., 1992). One possible reason for the death of the mouse embryos could be the lack of expression of imprinted alleles. The viability of *MET1a/s* plants and the gametes they produce suggests that hypomethylation in these plants does not result in the lack of expression of essential genes. Hence, it is possible that a less complex relationship between methylation and imprinted gene expression has evolved in plants, with hypomethylation promoting biallelic expression of imprinted genes (Spielman et al., 2001). Indeed, in maize a number of imprinted genes exhibit a correlation between repression and parent-specific hypermethylation (Alleman and Doctor, 2000).

A further consideration is that demethylation is not complete in *MET1a/s* plants, (Finnegan et al., 1996). MET1 activity is unlikely to be fully removed in *MET1a/s* plants due to the inactivity of the 35S promoter in certain tissues, and therefore some MET1 catalysed methylation maybe maintained (Wilkinson et al., 1997). Furthermore *A.thaliana* has several potential methyltransferases, including the chromomethylases, the *DRM* class and other members of the *MET1* methyltransferase gene family, whose activity is probably not affected in *MET1a/s* plants (Genger et al., 1999). Indeed the methylation of the *SUPERMAN* (*SUP*) locus by the CMT3 enzyme (a key determinant in non-CpG methylation) is enhanced in *MET1a/s* plants (Jacobsen and Meyerowitz, 1997; Lindroth et al., 2001; Bartee et al., 2001). This partial demethylation (and hypermethylation) could affect gametes and individual sequences unequally and this may lead to some aspects of development being affected more than others in [*MET1a/s* X *MET1a/s*] seed (e.g. CE development hindered more than PE proliferation).

3.3.4 Are imprinted genes essential for development?

Jaenisch (1997) proposed that the removal of imprints or of imprinted genes themselves should have few developmental consequences, as they exist in 'paired sets' of genes involved in the same pathway" (e.g. sets of growth promoters and inhibitors, as predicted by Haig and colleagues). Such theories are difficult to test in mice as embryos with reduced methylation die during gestation (Li et al., 1992). However, the results from the crosses with *MET1a/s* plants support the theory outlined above. Although [*MET1a/s* X *MET1a/s*] seed exhibit unpredicted phenotypes, their development was more comparable to a [2x X 2x] seed than the progeny from crosses with only one *MET1a/s* parent. In other words, removing methylation (and by inference imprinting) from both parental sides had less effect on seed development than if methylation was removed from only one parent. Unfortunately we have no evidence concerning the removal of the sets of genes per se; instead we propose that biparental hypomethylation in effect adds sets of antagonistic genes.

3.3.5 Parent-of-origin effects were observed in seed from crosses between hemi*MET1a/s* and wild type plants, regardless of whether they inherited the *MET1a/s* transgene

The purpose of the crosses with hemi*MET1a/s* plants was to test the post-fertilization effect of the *MET1a/s* transgene on endosperm and seed development. At the start of the experiments described in this Chapter we were concerned that any activity of the *MET1a/s* transgene within the developing seed could lead to the loss of methylation from incoming wild type chromosomes. This could lead to any parent-of-origin effects on seed development in crosses with *MET1a/s* and wild type plants being diminished. However, the complementary phenotypes in seed from the reciprocal crosses between *MET1a/s* and wild type plants (discussed previously) indicated that activity of the *MET1a/s* transgene was not leading to the loss of imprinting-associated methylation from the wild type chromosomes. In addition to this the crosses with hemi*MET1a/s* plants provided further

evidence that the *MET1a/s* transgene was not having a direct effect on seed development after fertilization.

Crosses between hemi*MET1a/s* and wild type 2x plants produced seed with similar (although weaker) phenotypes to crosses between *MET1a/s* and wild type plants. [hemi*MET1a/s* X 2x] seed had features of paternal excess (similar to a [2x X 4x] cross), while [2x X hemi*MET1a/s*] seed exhibited features of maternal excess (comparable to a [4x X 2x] cross). Strikingly a single class of seed, with respect to mature weight, germination rate, size of CE and number of PE nuclei was observed for each set of crosses, even though half the progeny inherited the *MET1a/s* transgene (Figures 3.11-3.14). This is consistent with imprinting-associated methylation being maintained on the wild type chromosomes, despite the presence of the *MET1a/s* transgene.

The reciprocal crosses between hemi*MET1a/s* and wild type 4x plants produced less inviable seed than the equivalent crosses with homozygous *MET1a/s* plants. As one of the differences between the crosses is that half the progeny in the hemi*MET1a/s* crosses do not inherit the transgene, it was proposed that this could be the reason for the variation in germination rate. However, when the viable seed from either a [hemi*MET1a/s* X 4x] or a [4x X hemi*MET1a/s*] cross were germinated and the seedlings tested, approximately half of the seedlings had inherited the transgene in each cross. If the transgene affected the rate of seed abortion we would have predicted that either less, or more, than half of the viable seed (depending on the action of the transgene) would have contained the transgene.

Therefore the difference in germination rates observed in the crosses with hemi*MET1a/s* plants may be viewed as a weaker parental excess phenotype. Indeed this fits with the observation above for seed from crosses between hemi*MET1a/s* and 2x wild type plants. Here the seed have a weaker parental excess phenotype with respect to mature seed mass and endosperm proliferation, compared to progeny from the same crosses with *MET1a/s* plants. One possible explanation is that hemi*MET1a/s* plants in general have higher levels of methylation in the genome than *MET1a/s* plants (Figure 3.10; Finnegan et al., 1996).

Therefore, the hypomethylated gametes produced by the hemi*MET1a/s* plants could have lost less imprinting-associated methylation. In this scenario some of the imprinted alleles contributed to the endosperm by the hemi*MET1a/s* plants could retain a degree of their wild type imprinting status, so that overall the imbalance of imprinted gene expression is less drastic, resulting in a weaker parental excess phenotype.

As only one class of seed is observed in the crosses between hemi*MET1a/s* and wild type 2x plants this could indicate that any variation in imprinting-associated methylation is spread throughout the genome of a hemi*MET1a/s* plant. In this case hypomethylated regions would be segregated relatively equally into the gametes. This could result in only slight differences between seed with respect to imprinted gene expression, and such small variation may not be detected in the phenotype by the methods used. However, the crosses between hemi*MET1a/s* and 4x plants produce seed that we predicted were being pushed towards an extreme (and lethal) imbalance of imprinted gene expression. Therefore, even slight variations in imprinted gene expression (resulting from differences between levels of imprinting-associated methylation) could mean the difference between life and death. Unfortunately, it is difficult to test different levels of methylation in the genome of seeds or differences in levels of imprinted gene expression. However, observations made during these experiments highlighted a potential factor, which could be tested, that may allow the manipulation of imprinting.

The crosses between hemi*MET1a/s* and 4x plants showed a large variation in germination rate between seed from different pods for the same cross. For example in the [4x x hemi*MET1a/s*] cross one pod produced no viable seed, whilst another pod gave seed with a germination rate of 69%. Although this could be a result of the random segregation of alleles that have lost the imprinting-associated methylation and wild type alleles, it was noticed that there appeared to be a correlation with the age of the plants used in the experiments (data not shown). If a 4x plant that had just begun to flower was crossed with a hemi*MET1a/s* paternal parent, this resulted in very few viable seed (less than 5%). However, if a 4x plant that had been flowering for a longer period of time (about two weeks) was used in the same experiment, this produced a higher percentage of viable seed

(over 50%). Similar results were obtained regardless of the age of the hemi*MET1a/s* paternal parent. This raised the possibility that tolerance of imprinting imbalance in the endosperm may change with the age of the plants used in the crosses. The mechanisms behind any changes in tolerance could provide us with tools with which to manipulate imprinting and therefore the experiments were repeated in greater detail and are addressed in Chapter 4.

3.3.6 Timing of DNA methylation associated with the parent-of-origin effects

The results from the crosses between *MET1a/s* or *hemiMET1a/s* plants and wild type plants are consistent with *MET1* having a role in the parent-of-origin effects, and by inference imprinting, in *A.thaliana*. However, the time point at which this imprinting-associated methylation is established and the mechanisms by which it is propagated, both during gametogenesis and in the developing seed, remain elusive.

In mammals imprints are reset during gametogenesis, which takes place in the developing embryo (Figure 3.15, reviewed in Arney et al., 2001, Surani et al., 2001). Genome wide demethylation occurs in the primordial germ cells (PGCs), and results in the erasure of the allele specific methylation patterns (Surani, 1998, reviewed in Arney et al., 2001). Imprints are then reset in the mature gametes and propagated throughout development of the organism. However, in plants the resetting of imprints may not be required as the endosperm, (which is thought to be the major target of imprinting, with only indirect effects on embryo development), does not contribute genetically to the next generation. Indeed the imprinted gene *MEA* was shown to be biallelically expressed in the globular embryo, but monoallelically expressed in the early endosperm, suggesting that maternal imprinting of the locus is restricted to the endosperm (Luo et al., 2000). In addition, plants are unlikely to undergo the global cycles of demethylation and remethylation observed in mammals, since the progeny from hemi*MET1a/s* plants do not regain

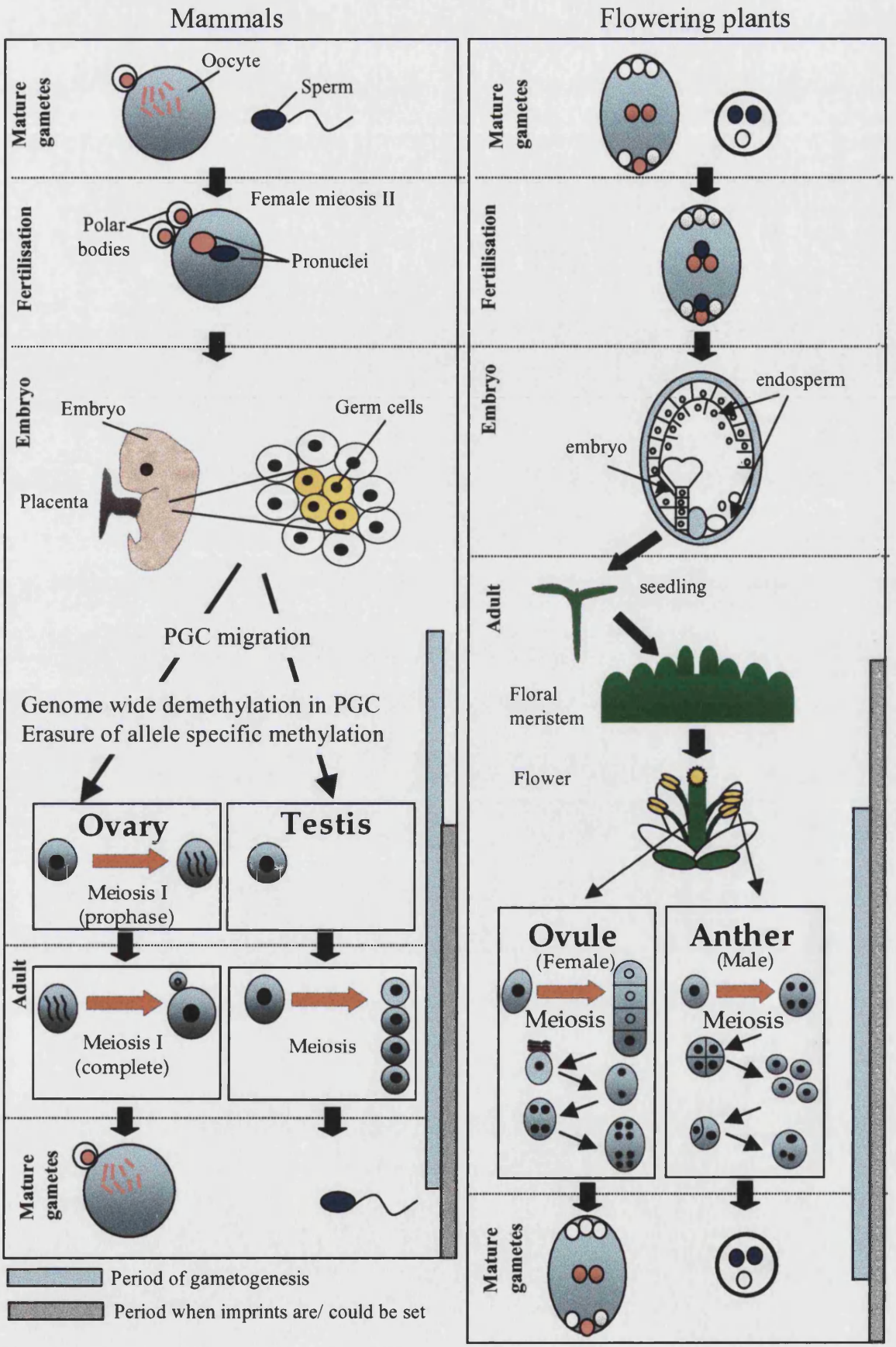


Figure 3.15
Reproduction in mammals and flowering plants. Imprints in mammals are reset during gametogenesis which initiates in the developing embryo. In plants imprints could be set at any time point between floral organ differentiation and fertilization. Adapted from Spielman et al., 2001.

wild type levels of methylation, even when they fail to inherit the *MET1a/s* transgene (Finnegan et al., 1996; Ronemus et al., 1996).

Unlike mammals, many flowering plants are hermaphrodites and therefore the imprints must be set following the separation of the precursors of male and female gametes. This could occur at any time point between stamen and carpel initiation and fertilization (Figure 3.15). Determining when MET1 catalysed imprinting-associated methylation is established could help pin point when imprints are set, even if methylation is not the primary imprinting mark in plants.

3.3.6.1 Establishing MET1 imprinting-associated methylation

Some evidence for the time point at which imprinting-associated methylation may be set came from the crosses between hemi*MET1a/s* and wild type plants. In these crosses each seed developed according to the methylation status of the parents, regardless of whether the seed inherited the transgene. One possible explanation for this is that at least some element of the parent-specific methylation is set prior to the first nuclear division of meiosis. Alternatively, the gametes themselves may not express MET1 but inherit MET1 protein from the diploid mother cells. In this scenario it would be the genotype of the parent plant, rather than that of the meiotic product, that is reflected in the seed phenotype.

A third possibility is that the epigenetic state of the DNA is important for the setting of imprinting. In other words, it could be the hypomethylated state of the DNA that is preventing imprinting-associated methylation being established and not the absence of active MET1 enzyme at a critical time point. For example imprinted genes could be methylated in the sporophytic tissues and it could be the sex-specific retention and maintenance of methylation that sets up imprints. Consequently, where DNA lacks methylation imprints can not be set because of the absence of any pre-existing methylation. Alternatively, methylation present on imprinted genes that enter gametogenesis could recruit chromatin remodelling complexes, which in turn could

further epigenetically modify the imprinted locus, perhaps by histone methylation. Sex-specific modifications could occur if different methylation recognition factors were expressed during male and female gameteogenesis.

The difficulty in distinguishing between the proposals is the lack of information on the wild type expression pattern of the MET1 protein. For example, if *MET1* is expressed during gametogenesis this would be evidence to contradict the proposal that the gametes acquire MET1 protein from the surrounding parental cells. Therefore the wild type expression profile of *MET1* was analysed and these experiments are described in Chapter 5.

The analysis of the results from the hemi*MET1a/s* experiments was complicated further by the growing evidence that the 35S promoter is not active in many of the tissues in which imprints could be set (Table 3.3). As the 35S promoter drives the expression of the *MET1a/s*, if the 35S promoter is not active then no *MET1a/s* RNA will be present enabling the translation of the MET1 protein. Theoretically, this could allow us to narrow the window in which imprinting-associated methylation is established. For example, activity of the 35S CaMV promoter in flowers and buds is confined mainly to the sporophytic vascular tissues (Wilkinson et al., 1997). Furthermore, there is no evidence for 35S activity in *A.thaliana* microspores or pollen (Wilkinson et al., 1997). This suggests that if *MET1* acted during floral development or gameteogenesis to establish and propagate imprinting-associated methylation it would **not** be prevented from doing so by the expression of the *MET1a/s* RNA in the *MET1a/s* plants. However, since we observed the parent-of-origin effects on seed development in the *MET1a/s* crosses, this indicates that the 'essential' action of the MET1 protein with respect to imprinting-associated methylation, which is lost in *MET1a/s* plants, does not occur during gametogenesis. However, *MET1* may still have an important role in the propagation of imprinting-associated methylation during gametogenesis, with the enzyme simply having no methylation template to replicate in *MET1a/s* plants.

Table 3.3
Activity of the 35S CaMV promoter in different plant tissues

Floral, gametophyte or seed stage	**Is the 35S CaMV promoter active?**	**In which tissues is the 35S CaMV promoter active ?**
Floral meristem	YES	35S expressed in all 4 whorls of the floral meristem (Mandel et al., 1992; Kritez et al., 1996; Lilegren et al., 1999)
Floral buds	YES	35S expression in *A.thaliana* confined to the vascular tissue of sepals, stem and stigma in buds (Wilkinson et al., 1997).
Mature flowers	YES	35S expression in *A.thaliana* almost completely confined to the vascular tissue of the stem, sepals, anther and filament. Some 35S expression noted in a few lines in the petals and stigma. (Wilkinson et al., 1997)
Pollen	NO	No 35S expression in *A.thaliana* pollen (Wilkinson et al., 1997). 35S promoter not active during microspore or pollen development in *Brassica napus* (Custers et al., 1999).
Ovule	NO	No data
Early seed development	NO	The 35S promoter is not expressed in the embryo until the heart-torpedo stage or in the syncytial endosperm (Boisnard-Lorig et al., 2001).

In contrast, there is evidence that the 35S transgene is active in all four whorls of the organ primordia in the floral meristem (Mandel et al., 1992; Kritez et al., 1996a; Liljegren et al., 1999). Therefore the MET1 protein could be important in the establishment (or propagation) of imprinting-associated methylation during floral organ differentiation. Again the determination of the wild type expression pattern of the MET1 protein will allow further analysis of this hypothesis.

3.3.6.2 Propagation of *MET1* imprinting-associated methylation in the seed

The parent-of-origin effects observed in the seed from reciprocal crosses between *MET1a/s* and wild type plants suggest that imprinting-associated methylation patterns (or imprints) are maintained on the chromosomes inherited from the wild type parent. As the 35S CaMV promoter is not active in either the embryo or the endosperm during early seed development (Boisnard-Lorig et al., 2001) MET1 may still have a role in propagating imprinting-associated methylation, even in seed containing a *MET1a/s* transgene. Therefore the wild type expression profile of MET1 will also be addressed in Chapter 5.

3.3.7 A model for the effect of global DNA hypomethylation on parental imprinting in *A.thaliana*

The results outlined in this chapter support the proposition that *MET1* catalysed DNA methylation as has an important role in the parent-of-origin effect in *A.thaliana*, consistent with an essential role for methylation in the parental imprinting mechanism in flowering plants. Global hypomethylation appears to derepress genes contributed to the seed by the polar nuclei that would normally be active only in the male genome, and derepress genes contributed by the sperm that would normally be female-specific. This has an effect of 'paternalizing' the female genome and 'maternalizing' the male genome (Figure 3.16 A). The phenotypic consequences are shown in Figure 3.16 B. We have shown that it is possible through uniparental methylation to modify seed development and ultimately seed size, most likely through lifting the silencing on parentally imprinted genes. Not only does this provide us with a tool with which to manipulate seed development, it also gives us an insight in to the mechanisms of genomic imprinting in plants.

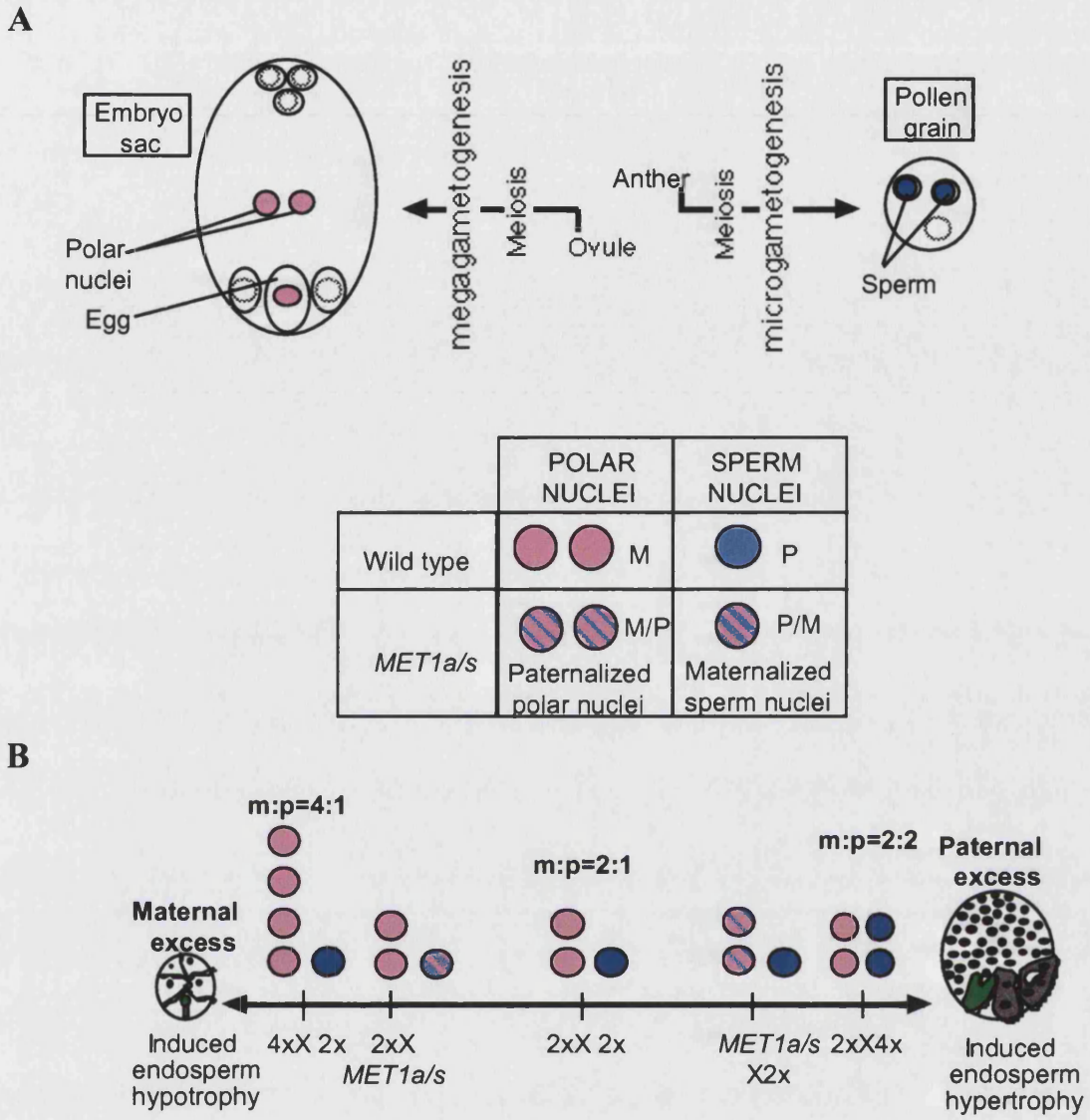


Figure 3.16

Model of the effect of global DNA hypomethylation on parental imprinting in *A.thaliana*. (A) Normally endosperm contains a ratio of 2 maternal genomes, contributed by the polar nuclei, to 1 paternal genome, contributed by the sperm. In maternal genomes, maternal-specific imprinted genes are active, while paternal-specific genes are repressed. Imprinted genes contributed by the paternal genome have a complementary expression pattern. When maternal genomes are contributed by the *MET1a/s* parent, the paternal-specific genes are expected to be largely derepressed, producing a 'paternalized' genome. Similarly a *MET1a/s* pollen parent is expected to contribute a 'maternalized' genome. (B) Interploidy crosses (e.g. [4x X 2x] or [2x X 4x] result in seeds with extra maternal or paternal genomes, and therefore extra doses of active maternal or paternal alleles of imprinted loci. Maternal or paternal excess has dramatic and complementary effects on seed development, resulting in small seeds with small endosperms and large seeds with overgrown endosperms, respectively (described by Scott et al., 1998). A diploid *MET1a/s* parent does not contribute extra genomes but appears to contribute extra doses of active maternal- or paternal-specific genes, resulting in phenotypes similar to those produced by parental genomic imbalance.

Chapter 4

A screen for T-DNA insertions in putative DNA methyltransferases in *A.thaliana*

4.1 Introduction

The experiments outlined in the previous Chapter were based on the proposal that if methylation had a role in the parent-of-origin effects, removing methylation from one parent in a cross would phenocopy the addition of extra parental genomes to the endosperm. Ideally, the hypomethylated plants used in the experiments should have been homozygous for a null allele in any methyltransferase potentially involved in the parent-of-origin effects. However, at the time the experiments were designed a total of 4 sequences that encoded putative DNA methyltransferases were identified in the *A.thaliana* genome. This number continued to rise until the complete genome sequence became available in 2000 (The *Arabidopsis* Genome Initiative, 2000). The *A.thaliana* genome is now predicted to encode a total of 9 putative DNA methyltransferases.

It was conceived that if DNA methylation had a role in the parent-of-origin effects then this could involve any of these potential DNA methyltransferases. However, at the beginning of the work outlined in this thesis null alleles had been reported for only one of these sequences, *CMT1* (Heinkoff and Comai, 1998). Sequencing of the *CMT1* locus in the commonly used ecotypes Ler, No-0 and RLD, had showed the genes to harbour a 4.7kb retrotransposon, that following RNA splicing results in the production of a truncated protein. This truncated protein is predicted to be inactive due to the absence of part of the downstream catalytic domain. Furthermore, in the Col ecotype the *CMT1* locus was shown to have multiple alternative splice sites, which results in only a quarter of the mRNA encoding for a functional protein. Since both the Col and Ler ecotypes had been shown to exhibit strong parent-of-origin effects in interploidy crosses (Scott et al., 1998) it was unlikely that *CMT1* encodes the primary factor involved in this phenomenon. For this reason, and due to time limitations, the work described in this Chapter concentrated on other putative DNA methyltransferases.

Therefore the objective of the work described in this Chapter was to isolate null alleles for all the other putative DNA methyltransferases in *A.thaliana*. The ultimate aim was to use each line in crosses with wild type plants, (as described in the previous Chapter), to study the role of each gene in the parent-of-origin effects. Although we were already testing the role of the *MET1* encoded enzyme in the parent-of-origin effects using the *MET1a/s lines,* due to the problems envisaged (and outlined in Chapter 3) with using an antisense line, *MET1* was also included as target sequence in the screen for null alleles. An efficient method to knockout plant gene expression is by insertion of T-DNA into, or near to, the gene sequence. Therefore, we proposed to systematically screen T-DNA lines to isolate null alleles in all sequences in *A.thaliana* that were predicted to encode DNA methyltransferases.

4.2 Results

4.2.1 The screen for T-DNA insertions in putative DNA methyltransferases in the Feldmann T-DNA lines

The first screen was conducted on the Feldmann T-DNA lines (McKinney et al., 1995). These lines were available as pooled DNA from approximately 6000 different T-DNA insertion lines, allowing a large amount of different insertions to be screened in a short period of time. The first set of DNA was provided as 6 aqueous samples, each containing DNA from approximately 1000 different T-DNA lines. This was screened as described below. If one of these 6 pools was positively identified as containing a T-DNA insertion in the required target gene then smaller DNA pools could then be screened until an individual T-DNA line was isolated. To screen the Feldmann lines for T-DNA insertions required the following steps.

1) The identification of potential DNA methyltransferase sequences in the genome of *A.thaliana*
2) The design of gene specific primers for each target sequence. These were to be used in conjunction with T-DNA border primers (provided with the T-DNA pools, McKinney et al.,1995) to screen the T-DNA pools via PCR amplification. Primer sequences were LB (GATGCACTCGAAATCAGCC AATTTTAGAC) and RB (GCTCATGATCAGATTGTCGTTTCCCGCCTT).
3) The design and synthesis of gene specific probes. Due to random priming reactions and the low representation of the DNA from any single T-DNA insertion line in the T-DNA pools it was recommended that the PCR products were hybridised to a target gene-specific probe (McKinney et al.,1995; R.Scholl, personal communication).
4) The systematic screen of the T-DNA pools for insertions in the target sequences.

4.2.1.1 The identification of potential DNA methyltransferase sequences in the genome of *A.thaliana*

At the beginning of the experiments described in this Chapter a total of 4 sequences were identified in the *A.thaliana* genome that were predicted to encode DNA methyltransferases (Table 4.1). Two sequences, *MET1* and *CMT1*, were identified from the literature (Finnegan et al., 1993; Heinkoff et al., 1998). The remaining two potential methyltransferases were identified via BLAST searches with *MET1* and *CMT1* used separately as the query sequence.

Table 4.1

Predicted DNA methyltransferase sequences in the *A.thaliana* genome. Each sequence is shown with its reference name and Genbank accession number. MET-like and MET2 were isolated via BLAST searches, however they have since been referred to in the literature with different reference names (column 2).

Reference name for sequence (1998)	Current reference name for sequence (2002)	Genbank Accession number
MET 1 (Finnegan et al.,1996)	*MET1*	L10692
MET1-like	*METIIa* (Genger et al.,1999)	AF138283
MET 2	*CMT 2* (Rose et al.,1998; Genger et al.,1999)	AL021711
CMT1 (Heinkoff and Cormai, 1998)	*CMT1*	U53501

MET1, *METIIa* and *CMT2* were chosen for the screen. *CMT1* was not chosen for the reasons described previously. *METI* and *METIIa* show a high level of homology, both within the N-terminal region (70% amino acid identity) and within the C-terminal catalytic domain (80% amino acid identity) (Genger et al., 1999). It was conceived that due to this high level of homology the screen for T-DNA insertions in *MET1* could identify insertions in *METIIa* by mistake, or visa versa. However, as no null alleles were available at the time for either sequence this was not considered to be a problem. Furthermore, the analysis of any lines identified as carrying a T-DNA in a target gene would confirm the exact point of insertion.

CMT2 is 60-80% identical to *CMT1* within the protein motifs, but shows only 30-70% identity with comparable motifs in the *MET1* and *METIIa* (Genger et al., 1999). Furthermore *CMT2* is over 25% longer than *CMT1*, (6410bp compared to 4048bp) as *CMT2* encodes for a longer amino-terminal domain. This domain shows no similarity to that of either *MET1* or *METIIa*. It was therefore concluded that the screen for T-DNA insertions in *CMT2* was unlikely to identify insertions in *METI*, *METIIa* or *CMT1*. Although, of course, it was planned to confirm this by detailed analysis of the insertion site of any lines identified in the screen.

4.2.1.2 The design of gene specific primers for each putative DNA methyltransferase

The screen of the T-DNA pools was a reverse-genetic PCR based approach (McKinney et al., 1995). The aim was to design primers that if used in conjunction with a T-DNA border primer would only give a PCR product if the pool contained DNA from a plant line in which a T-DNA had inserted in the target gene (Figure 4.1B).

In total, 4 gene specific primers were designed for each target sequence (Figure 4.1A). The AF and AR primers for each gene were designed to anneal at the 5' and 3' extremes of the predicted coding region, respectively. This was to ensure that where the T-DNA had inserted close to either the start or end of the gene then a PCR product would still be amplified. There was concern that if the T-DNA had inserted within the central region of the gene this would, for example in the case of the CMT2 sequence, require the amplification of a product of over 3kb. It was conceived that this amplification could be outside the limits of the screen, due to the low representation of each T-DNA line in the pooled DNA samples. In order to minimise the chance of PCR amplification failure, the BF and BR primers were designed for each gene within the coding sequence. The sequences of all the primers are shown in Table 4.2.

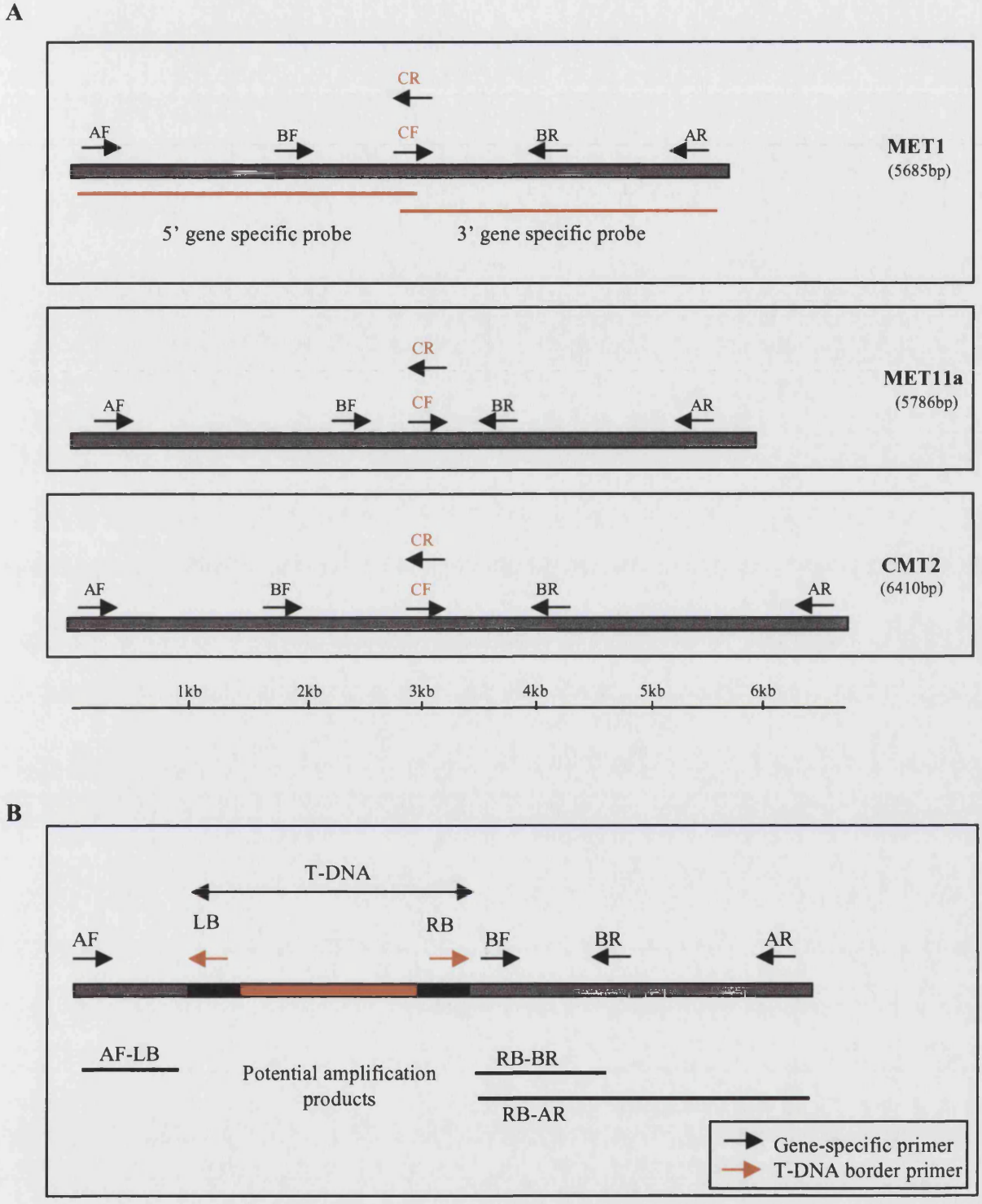


Figure 4.1
Diagrammatic representation of the reverse-genetic PCR screen for T-DNA insertions in target genes (A) The gene-specific primers (AF, BF,AR,CR) and probe primers (CF,CR) for all 4 target genes. As an example, the gene specific probes for *MET1* are shown. Note they incorporate most of the *MET1* coding sequence. (B) A representation of the possible PCR products amplified with gene-specific primers combined with T-DNA specific primers, if a T-DNA is inserted in the target gene.

Table 4.2

The target gene-specific primer sequences for each potential DNA methyltransferase.

Target gene	Primer name	Primer nucleotide sequence
MET1	AF	AAGCGAAAGAAGAGACCACTTCC
	BF	TGCAAGCAAATTGGAGGATAGG
	AR	TGCATTCCCAATCTGCCTGTGC
	BR	AGGTCCACCATTGATGAAGTCC
METIIa	AF	CTTTGGAGAGCAATGATGATCC
	BF	ATGGGAGGTGAGATTCTTGGC
	AR	AGTAATGCTAGAGGGCTGACG
	BR	AGTTCCAGTTTCTACTCCCTTCC
CMT2	AF	GAGAGATGAGTTTGAGAGATCC
	BF	CACTGGATCTGAATAATCCAACG
	AR	AGCTGAGGGTAAGTTGAATGCG
	BR	CTAGTTACAAACTCCCGAATGGC

4.2.1.3 The design and synthesis of gene specific probes

Gene-specific probes were designed for each target gene. The aim was to hybridize these probes to the PCR products (from reactions with one gene specific primer and one T-DNA primer) to identify target gene-specific bands. Two probes (a 5' probe and a 3' probe) were designed for each target gene (Figure 4.1A). In order to maximize the chance of successfully identifying the target gene specific products the probes were designed to incorporate the vast majority of the target sequence.

For this purpose the primers, CR and CF, were designed within the central region of each coding sequence. These were used in conjunction with the AF and AR primers, respectively, to amplify the probe fragments. The sequences of these primers are shown in Table 4.3. The PCR fragments of each probe were amplified and then cloned into pGEMT. The isolated vector was then used as the amplification product for the synthesis of the DIG labelled probe.

Table 4.3

The sequences of the primers used to amplify the genomic DNA to be used as gene-specific probes. In each case the primers used to amplify the 5' probe fragment are shown in the first row, followed by the primers used to amplify the 3'probe fragment of the same gene.

Target gene	Primer name [1]	Probe primer name	Probe primer nucleotide sequence	Size (kb)[2]
MET1	AF	CR	CCAAATCAGCCTTTCTAGATTCC	3.2
	AR	CF	TATAGGCCTGAGGATGTTTCTGC	2.5
METIIa	AF	CR	GCCTTTTCTTCTGAAATGTCCTCG	3.1
	AR	CF	AACTGACAAGGTTTTATAGGCCC	2.3
CMT2	AF	CR	GGATTCTGTAGGTACCACATCGG	3.1
	AR	CF	CAACAAGCTTGAGTGTTGTGCCG	3.2

[1]Primer sequence shown above in Table 4.2
[2]Size of PCR product to be used as gene-specific probe

4.2.1.4 The systematic screen of the Feldmann T-DNA lines for insertions in the target sequences that encode putative DNA methyltransferases.

Each of the 6 T-DNA pools was systematically screened for a transposon insertion in each of the 3 target gene sequences. This involved the PCR amplification of a sample of pool DNA with each gene-specific primer combined separately with each T-DNA border primer. This was a total of 8 reactions per pool per gene. The PCR products were then hybridized to gene-specific DIG labelled probes. If a pool contained DNA from a plant line with a T-DNA inserted in the target gene, then we expected to observe gene-specific PCR products (Figure 4.1).

In total, approximately 6 000 T-DNA lines (6 pools) were screened for insertions in the 3 target genes. However, each screen showed a high level of non-specific hybridization. Figure 4.2 shows the PCR products from the same set of 8 reactions for the *CMT2* sequence (each gene specific primer combined with each T-DNA primer) on 2 different pools of DNA, hybridized to the same *CMT2* gene-specific probe. The patterns of hybridization are almost identical for each pool. It is therefore unlikely that these amplification products resulted from reactions between gene-specific primers and T-DNA primers that had inserted within the target sequence.

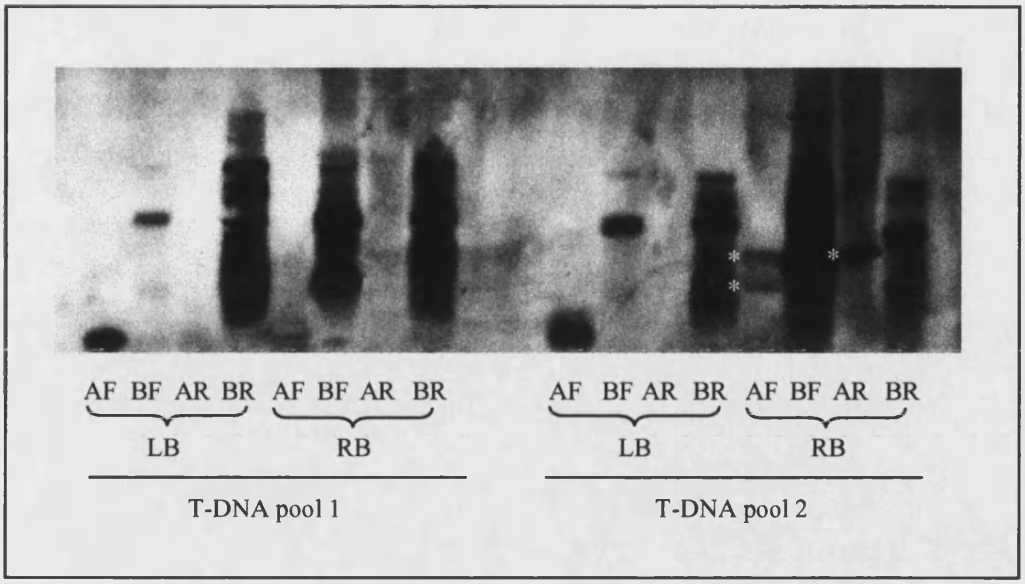


Figure 4.2
The PCR screen of the Feldmann T-DNA pools 1 and 2 for a T-DNA insertion in the *CMT2* target sequence. This shows a southern blot of the PCR products from the amplification between the *CMT2* gene-specific primers (AF, BF, AR and BR) and the T-DNA border primers (LB and BR), hybridized to the *CMT2* 5' gene-specific probe. The set of 8 reactions on the left were conducted on DNA from pool 1 and the set of reactions on the right were carried out on DNA from pool 2. The white stars highlight bands in the AF/RB and AR/RB lanes that are specific to the amplification reactions with DNA from pool 2.

A few pool specific bands were observed (highlighted in Figure 4.2 by a white star). In some cases these were very faint, (Figure 4.2 pool2 AF-RB) whereas we might expect more intense bands if they were PCR products from a T-DNA insertion reaction. In other cases these bands were intense (Figure 4.2 pool2 AR-RB reaction) but were not reproduced when the screen was repeated (data not shown). These results were typical of the other screens and no definite gene-specific bands were isolated in a single pool for all the 3 target genes.

4.2.2 The second screen of T-DNA lines for insertions in putative DNA methyltransferases

As the screen for T-DNA insertions in target genes in the Feldmann lines yielded no positive results we proposed a second screen for T-DNA insertions in putative DNA methyltransferases sequences. This second screen was performed solely at Ceres.

4.2.2.1 The identification of target sequences for the screen

Since the design of the Feldmann T-DNA line screen, an additional 2 sequences had been identified as putative DNA methyltransferases (Table 4.4). *METIII* encodes a protein that is 80% identical to the *METIIa* protein (Finnegan and Kovac, 2000). However in the ecotype Col, *METIII* encodes a truncated protein that is predicted to be inactive (Genger et al., 1999). Therefore for the same reasons discussed for *CMT1* *METIII* was not chosen as a target gene for the second screen.

METIIb encodes a protein that is over 90% identical to *METIIa* at the amino acid level (96.8% within the methyltransferase domain) (Finnegan and Kovac, 2000). Therefore it was a strong possibility that any screen with either *METIIa* or *METIIb* could identify a T-DNA insertion in the other sequence. However, as outlined before, this was not considered as a problem as no T-DNA insertion lines were available for either gene at the time and further analysis of any lines would easily confirm the insertion site of the T-DNA.

Therefore a total of four target sequences were chosen for this second screen and those were, *MET1*, *METIIa*, *METIIb* and *CMT2*. As the process was conducted solely at Ceres the information on the lines screened, the methods used and the primers designed have not yet become available.

Table 4.4
Additional predicted DNA methyltransferases in the genome of *A.thaliana*

Reference name of predicted DNA methyltransferase sequence	**Genbank accession number**
METIIb (Genger et al.,1999)	AC005359
METIII (Genger et al.,1999)	AL049656

4.2.2.2 The isolation of lines with T-DNA insertions in potential DNA methyltransferase sequences

In total T-DNA insertions were found in 3 of the target sequences. Insertions were identified in *MET1* (1 line) *METIIa* (3 different lines) and *METIIb* (1 line). No lines were identified with a T-DNA insertion in the *CMT2* sequence. The work was conducted at Ceres and no further information is currently available for these insertion lines.

4.3 Discussion

4.3.1 The Feldmann line screen identified no T-DNA insertions in the target genes

The aim of the work described in this Chapter was to isolate T-DNA insertion lines for all the predicted functional DNA methyltransferases in *A.thaliana*. The screen of the Feldmann T-DNA pools failed to identify any lines with a T-DNA insertion in the 3 target genes. It is possible that the pools did not contain DNA from plants carrying T-DNA insertions in the target sequences. Indeed, combined the pools contained DNA from approximately 6000 different T-DNA lines, with on average 1.5 T-DNA insertions per line (McKinney et al., 1995). Thereby, the screen used approximately 9000 insertions, while the *A.thaliana* genome contains 25, 498 genes (The *Arabidopsis* Genome Initiative, 2000). This is approximately only a 1 in 3 chance of a hit, even without taking into consideration insertion of the T-DNA into non-coding sequences. Furthermore, at least one other group has reported that they were unable to isolate a T-DNA insertion in the *CMT2* gene in the same T-DNA pools (McCallum et al., 2000).

It is possible that the T-DNA pools screened did contain lines with T-DNA insertions in at least some of the putative DNA methyltransferases, but that the gene-specific/T-DNA bands were not observed due to the very high level of non-specific hybridisation. In these experiments the probes were labelled with DIG UTP, as this was considered less harmful to the researcher than the more conventional P^{32} method. Although routinely used for other hybridizations the DIG UTP method may not have been sensitive enough to distinguish a single band from the other non-specific PCR products. It was concluded that if the experiments were repeated the screen should be expanded to test a larger number of T-DNA lines and the gene-specific probes labelled with P^{32}.

However, the experiments were extremely time consuming and at the time when the conclusions above were drawn, the *MET1a/s* crosses (outlined in the previous section) were yielding some very exciting results. Furthermore, the opportunity arose to collaborate with a company (Ceres) that were already routinely screening T-DNA

lines. Therefore, we decided to prioritise the *MET1a/s* experiments and not continue with the screen of T-DNA lines for target genes ourselves.

4.3.2 The Ceres screen

The screen of T-DNA lines for insertions in putative DNA methyltransferases conducted at Ceres was more successful. However, these lines were not received in time for them to be analysed for any parent-of-origin effects on seed development (in crosses with wild type plants). Indeed before such experiments are carried out each line needs to be tested to ensure that the insertion of the T-DNA results in a null allele of the target gene. The required characterisation is currently being carried in our group.

4.3.3 Mutations in potential DNA methyltransferases

The study of plant DNA methyltransferases has been extremely productive since the first plant methyltransferase was identified in 1993 (Finnegan et al., 1993). There are now a total of 9 putative methyltransferases identified in the completed *A.thaliana* genome sequence. A number of mutations have been isolated and characterised in a proportion of these sequences (Table 4.5). Of particular interest are the MET1 (*ddm2-1*) mutations identified by E.Richards group (quoted in Finnegan and Kovac, 2000). The *ddm2-1* line was kindly donated by E.Richards and work with these plants is currently underway within our group. It is hoped that by using these lines in crosses with wild-type plants and studying the parent-of-origin effect on seed development we will be able to gather further evidence to support *MET1* as having an important role in plant imprinting.

MET1 belongs to a small multigene family with 4 members, which includes *METIIa*, *METIIb* and *METIII* (Finnegan and Dennis, 1993; Genger et al., 1999). All of the 4 characterised sequences arose from the same ancestral gene by a series of duplication events (Finnegan and Kovac, 2000). Despite the high level of homology between *MET1* and the other members of the gene family (up to 80% amino acid identity in the methyltransferase domain) the expression of *METIIa* and *METIIb* was not affected in the *MET1a/s* plants (the effect on *METIII* expression was not reported) (Finnegan and

Kovac, 2000). As the parent-of-origin effects observed in crosses between wild-type and *MET1a/s* plants were not as drastic as predicted if all imprinting was relaxed (in *MET1a/s* plants) then it remains a distinct possibility that the other members of the *MET1* gene family could have a role in the parent-of-origin effects, and by inference imprinting.

In order to test this proposal lines carrying a *METII antisense* (*MET11a/s*) transgene were kindly donated by J.Finnegan. The *METII a/s* lines were designed prior to the identification of the second *METII* gene, *METIIb*. Nevertheless, the lines were predicted to down regulate the expression of both *METIIa* and *METIIb* due to their high levels of sequence homology (Finnegan and Kovac, 2000, J.Finnegan personal communication). However, these lines were subsequently found not to down regulate the expression of either of the endogenous gene products (METIIa and METIIb) (J.Finnegan personal communication). Therefore, obviously these lines could not be used to test the effect of reducing METIIa/b catalysed methylation on the parent-of-origin effects in *A.thaliana*.

The role of DNA methyltransferases from other gene families, in the parent-of-origin effects in *A.thaliana* also remains an intriguing possibility. Since both screens were designed a third chromomethylase, *CMT3* has been identified (McCallum et al., 2000; Bartee et al., 2001; Lindroth et al.,2001). One proposed role for this enzyme is to add CpNpG methylation on to heterochromatic regions (Bartee et al.,2001; Bartee et al.,2001a,b; Lindroth et al.,2001; Jackson et al.,2002). This could lead to the formation of a highly stable heterochromatic structure in processes such as parental imprinting. Although a number of studies have indicated that plants homozygous for a *CMT3* loss of function allele do not exhibit any developmental abnormalities, none have looked at parent-of-origin effects on seed development (McCallum et al.,2000; Bartee et al.,2001; Lindroth et al.,2001).

Perhaps of further interest is that no loss-of-function alleles have yet been identified in the *CMT2* locus, by either of the 2 screens described in this Chapter, or in different screens conducted by other groups (McCallum et al., 2000). This may suggest that *CMT2* encodes for an essential gene.

A third class of methyltransferase, the *Domains Rearranged Methyltransferases* (*DRM*) class, has also been identified in *A.thaliana* since the T-DNA screens described in this Chapter were designed (Cao et al., 2000). Although no null alleles of either of the predicted *DRM* genes (*DRM1* and *DRM2*) have yet been reported, their predicted *de novo* methyltransferase activity also makes them interesting candidates for a role in the parent-of-origin effects. For this purpose the expression profile of DRM2 during seed development and gametogenesis is studied in Chapter 5.

Table 4.5

Reported mutations in putative DNA methyltransferases

Putative DNA methyltransferase	Null alleles or antisense lines available
MET1	*METIa/s* (Finnegan et al.,1996; Ronemus et al.,1996) *ddm2* E.Richards (quoted in Finnegan and Kovac, 2000) *MET1* T-DNA insertion line (isolated by Ceres)
METIIa METIIb	*METIIa/s* Designed against METIIa, but due to the high homology between METIIa and METIIb should be effective against both mRNAs (J.Finnegan personal communication; Genger et al.,1999). *METIIa* 3 T-DNA insertion lines (isolated by Ceres) *METIIb* 1 T-DNA insertion line (isolated by Ceres)
CMT3	Allele encoding a truncated product (McCallum et al.,2000) Loss-of-function alleles isolated (Bartee et al.,2001) Loss-of-function alleles isolated (Lindroth et al.,2001)

4.3.4 Future work

The role of DNA methyltransferases other than the *MET1* encoded enzyme in the parent-of-origin effects in *A.thaliana* remains to be tested. Although the experiments outlined in Chapter 3 supported MET1 as having an essential role in imprinting, it is possible that the enzyme works in conjunction with other methyltransferases in this process.

The T-DNA insertion lines isolated in the screen conducted by Ceres, once characterised, could be used to study the role of METIIa and METIIb in the parent-of-origin effects, by crossing homozygous lines with wild type plants and studying the developing seed. The role of CMT3 could also be tested with the use of some of the many reported null alleles. Other T-DNA pools could also be screened for insertions in sequences, such as *CMT2* and *DRM2*, in which null alleles are not yet available. The role of MET1 in the parent-of-origin effects could also be clarified by repeating the experiments in Chapter 1 with either the *ddm2/ddm2* line or the *MET1* T-DNA insertion line isolated in the screen conducted by Ceres.

Furthermore, the expanding field of epigenetics has highlighted the potential role of factors other than methyltransferases in the process of imprinting. Indeed the recent link made between histone methylation and DNA methylation in the fungus *Neurospora crassa* (Tamaru and Selker, 2001) and *A.thaliana* (Jackson et al., 2002) suggests a multi-tier mechanism by which certain genes (for example imprinted alleles) could be condensed into stably silenced heterochromatin. Therefore, to comprehensively study the epigenetic mechanisms behind the parent-of-origin effects in *A.thaliana* the work should be extended to encompass factors such as histone methyltransferases, histone deacetylases and other putative components of the silencing machinery.

Chapter 5

The effect of parental plant age on the parent-of-origin effects on seed development

5.1 Introduction

The crosses between hemi*MET1a/s* and 4x plants (reported in Chapter 3) produced seed that showed a large variation in the rate of germination between individual pods of the same cross. Preliminary observations suggested that the rate of germination of the seed from these crosses might be positively correlated to the age of the maternal plant. One possible explanation is that this reflected an increased tolerance to an imbalance of parental genomes in seed with an older maternal parent.

However, these preliminary assessments were made on a small sample size and no record was made of the exact age of the plants. Also, because of the small sample size, the effect of paternal plant age on the seed viability could not be ruled out. Therefore we proposed to study in greater detail the effect of parental plant age on seed viability in the progeny from crosses between hemi*MET1a/s* and 4x wild type plants.

If parental age is a factor in seed viability in these crosses, this could reflect a general effect of plant age on seed development (and perhaps on the plant imprinting system). However, the effect of plant age may only have been observed in the progeny from crosses between hemi*MET1a/s* and 4x plants because the seed are delicately balanced between viability and abortion, and therefore even slight changes in development could have a dramatic impact on the seed phenotype (Section 3.3.5). In order to test if seed development in general is affected by plant parental age the crosses described in Section 3.2.3 were repeated (including balanced crosses, interploidy crosses with plants with wild type methylation levels and crosses between hemi*MET1a/s* and 2X plants) and the effect of plant age on seed development measured as changes in mature seed mass.

5.2 Results

To examine if the age of the plants in a cross effects seed development we crossed plants at different developmental stages and tested if the resulting seed showed any variation in seed mass or viability.

5.2.1 The standardization of the age of the parental plants

The age of a plant does not necessarily correlate with its developmental stage. For example, flowering time can vary greatly depending on the ecotype used and the environmental conditions. A further complication arose when using hemi*MET1a/s* plants and 4x plants as they exhibited a significant delay in flowering (of up to 3 weeks) compared to the 2x plants. Due to the inherent plasticity of plant development we decided to classify plant age not in terms of time elapsed since germination or sowing, but in terms of the number of flowers produced on the primary bolt. To maximise the growth of the primary bolt for the experiments all other shoots produced by the plants were removed. This also further standardised the experiments as hemi*MET1a/s* and 4x plants normally produced many more shoots than the 2x plants.

3 arbitrary stages of development: 1, 2 and 3 were used in the experiments. The early stage (stage 1) included flowers 10 to 15 (where flower 1 is the first flower produced). The first few flowers were not used as the 4x maternal plants had to be emasculated since no male sterile lines were available. Emasculation of the first emerging flowers can be extremely difficult, due to the short length of the primary bolt, and can result in damage to the plant. Stage 2 included flowers 28 to 33, and the late stage (stage 3) included flowers 58 to 63, which on average were the last flowers produced by the primary bolt before the plant went to seed.

5.2.2 The design and execution of crosses to test the effect of parental plant age on seed development

The observation which led to the experiments described in this Chapter was that the seed from crosses between hemi*MET1a/s* and 4x plants showed a large variation in viability, with preliminary experiments indicating that maternal plant age may be a controlling factor. To test this in greater detail the crosses between hemi*MET1a/s* and 4x plants were repeated with plants at standardised developmental stages as described below. It was proposed that the age of the plants in a cross could affect wild type seed development, therefore the experiment was extended to study seed from [2x X 2x] and [4x X 4x] crosses. However, the crosses between hemi*MET1a/s* and 4x plants vary from these wild type crosses in 2 ways: 1) one of the parental plants is hypomethylated and 2) an unbalanced number of parental genomes is added to the endosperm. Thus, to test if the effect of parental age is due to the hypomethylation reciprocal crosses were also carried out between 2x and hemi*MET1a/s* plants. To examine if the unbalanced ratio of parental genomes in the endosperm produced the parental age effect on seed development reciprocal interploidy crosses were carried out between 2x and 4x plants.

Therefore, to study if the age of the plants in a cross affects seed development, crosses were carried out between maternal and paternal plants at the 3 chosen developmental stages (outlined above). For example, stage 1 maternal plants were crossed separately with paternal plants at developmental stages 1, 2 and 3. The mature seed from each cross was collected, weighed and in the case of the crosses between *hemiMET1a/s* and 4x plants, subjected to a germination assay. This data was then analysed to test if parental plant age had a significant effect on mature seed size and/or viability. An example table of the data is shown in Table 5.1. The seed mass data for the remaining 8 crosses is provided in Appendix A.1-A.8. The germination assay data is listed in Appendix B.

Table 5.1

The mature mass of seed from [2x X 2x] crosses with plants at different developmental stages. The data has a normal distribution (A^2=0.318, $d.f$=20, p=0.513) with the Anderson-Darling Normality Test (Sokal and Rohlf, 1995).

Developmental stage of the 2x maternal plant	Developmental stage of the 2x paternal plant	Seed mass in μg[1]
1	1	30
1	1	28
1	2	28
1	2	31
1	3	30
1	3	23
2	1	34
2	1	36
2	2	32
2	2	34
2	3	33
2	3	37
3	1	34
3	1	38
3	1	38
3	1	35
3	2	34
3	2	43
3	3	36
3	3	38
3	3	37

[1] The mean mass of seed from a single pod

5.2.3 The effect of parental plant age on seed development

5.2.3.1 The effect of parental plant age on mature seed mass

To determine the impact of parental age on mature seed size, each set of seed masses was subjected to a 2-way analysis of variance (ANOVA) test (Sokal and Rohlf, 1995). The test could be used as each data set was shown to have a normal distribution using the Anderson-Darling method (Table 5.1, Appendix A.1-A.8). The 2-way ANOVA method allowed the effect of maternal age, paternal age and any interaction between these two factors to be analysed simultaneously. The results for each cross are shown in Table 5.2.

Table 5.2
The effect of parental plant age on mature seed size

Cross	Does seed mass increase with maternal plant age?[1]	Between which maternal ages is the increase in seed mass significant? [2]	Does seed mass increase with paternal plant age?[1]
2x X 2x	YES (F=17.09,$d.f$=2, p<0.0001)	1 and 2 (T=3.63, p=0.0089) 1 and 3 (T=5.82, p=0.0002)	NO
2x X 4x	YES (F=8.38,$d.f$=2, p=0.005)	1 and 3 (T=4.01, p=0.0039) 2 and 3 (T=2.75,p=0.0410)	YES (F=4.86, $d.f$=2, p=0.026) Between paternal stages 1 and 3 (T=2.91, p=0.0308) [2]
2x X hemi*MET1a/s*	NO		NO
4x X 2x	YES (F=4.05, $d.f$=2, p=0.039)	1 and 3 (T=2.82, p=0.0329)	NO
4x X 4x	YES (F=24.46, $d.f$= 2,p<0.0001)	1 and 2 (T=3.05, p=0.0301) 1 and 3 (T=6.988, p=0.0001) 2 and 3 (T=3.458,p=0.0154)	NO
4x X hemi*MET1a/s*	YES (F=8.19,$d.f$=2, p=0.009)	1 and 3 (T=4.011, p=0.0077)	NO
hemi*MET1a/s* X 2x	YES (F=19.19, $d.f$=2, p=0.001)	1 and 3 (T=5.96, p=0.0009) 2 and 3 (T=4.99, p=0.0027)	NO
hemi*MET1a/s* X 4x	NO		NO
hemi*MET1a/s* X hemi*MET1a/s*	YES (F=6.35,$d.f$=2,p= 0.019)	1 and 3 (T=3.19, p=0.0269) 2 and 3 (T=3.16, p=0.0284)	NO

1 The seed mass data for each cross was analysed using the 2-way ANOVA test. Therefore the analysis of seed mass variation with respect to both maternal and paternal age came from the same statistical test.
2 If the 2-way ANOVA test showed there to be a significant difference in seed mass with an increase in parental plant age the data was analysed with the Tukey Simultaneous test. This identified between which parental developmental stages in a cross there was a significant increase in mean seed mass.

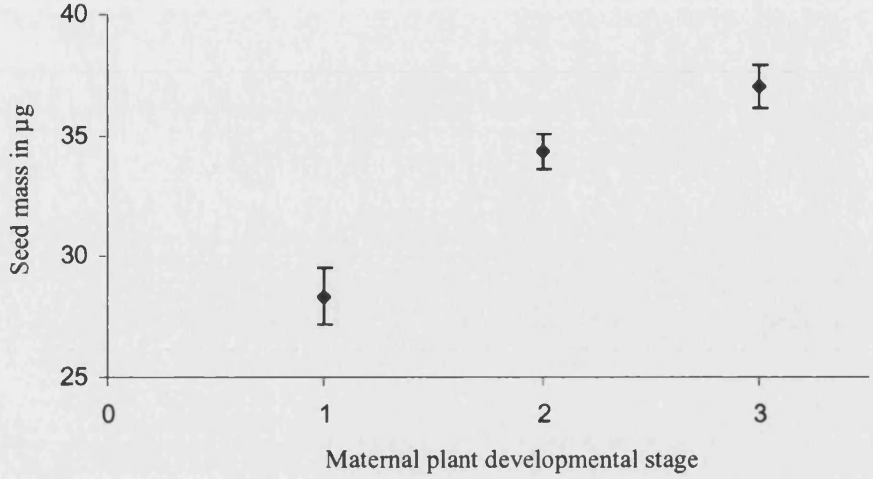


Figure 5.1

The increase in seed mass with maternal plant age in a [2x X 2x] cross. The average seed mass for each maternal stage (crossed with each paternal stage) is shown by the solid blue circle. The lines represent the standard error of the mean (s.e). The increase in seed size is significant between stages 1 and 2, (T=3.63, p= 0.0089) and stages 1 and 3, (T=5.8, p=0.0002) using the Tukey Simultaneous Test. There was no significant difference between seed from Stages 2 and 3.

Strikingly, in 7 out of the 9 sets of crosses the age of the maternal plant was shown to have a significant effect on mature seed size. In contrast, only the cross [2x X 4x], exhibited a significant effect on mature seed mass with respect to the age of the paternal parent. Graphical representation of the data showed that in these crosses as maternal age increased (and paternal age in the case of the [2x X 4x] crosses) so did the average mass of the seed (Figures 5.1 and 5.2).

The data was then subjected to a Tukey simultaneous test (Sokal and Rohlf, 1995) to determine if the variation in seed mass occurred between specific developmental stages. The results are shown in Table 5.2. In all 7 cases a significant difference was noted between the average seed masses from maternal developmental stages 1 and 3 (Figure 5.1 and 5.2A). The significant paternal effect on seed mass in the [2x X 4x] cross was shown to be between paternal developmental stages 1 and 3 (Figure 5.2B).

5.2.3.2 The effect of parental plant age on seed viability in crosses between hemi*MET1a/s* and 4x plants

The seed from the [4x x hemi*MET1a/s*] and [hemi*MET1a/s* x 4x] sets of crosses were assayed for their rate of germination (Appendix B). As these data sets were found not to have a normal distribution (with the Anderson-Darling method) the non-parametric Krustal-Wallis test (Sokal and Rohlf, 1995) was used to test if seed viability varied with the age of the plants in the cross.

In the cross [4x x hemi*MET1a/s*] seed viability was found to increase significantly with maternal plant age (H=8.78, $d.f$=2, p=0.012) (Figure 5.3A). There was no significant increase in the proportion of viable seed with an increase in paternal plant age. The reciprocal cross, [hemi*MET1a/s* x 4x], showed no variation in the number of viable seed, with respect to parental age (Figure 5.3B).

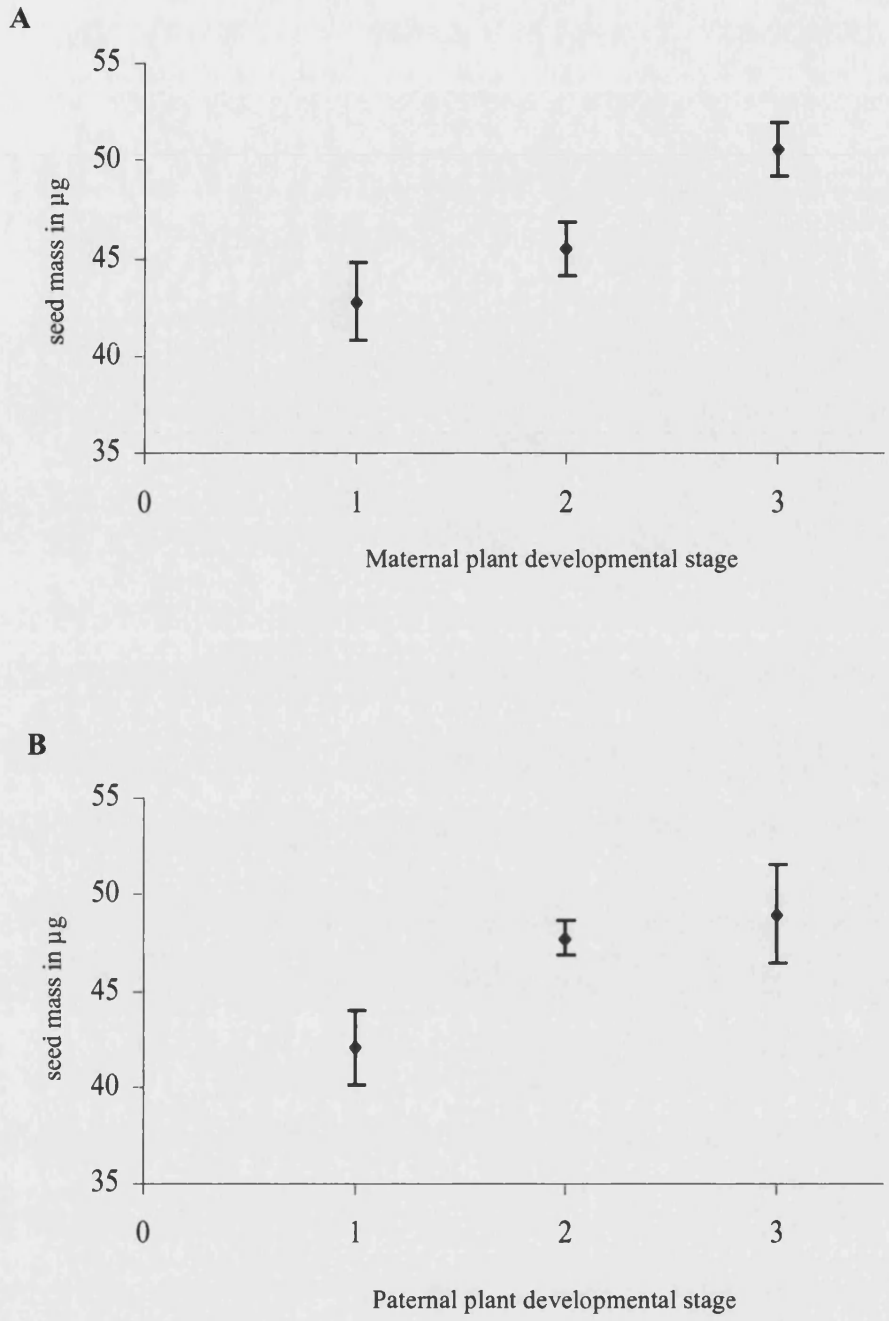


Figure 5.2

Increase in seed mass with maternal (A) and paternal (B) plant age in a [2x X 4x] cross.The increase in seed size was significant between maternal stages 1 and 3 (T=4.01, p=0.0039) and stages 2 and 3 (T=2.75, p=0.041)using the Tukey Simultaneous Test. The increase in seed size was significant between paternal stages 1 and 3 (T=2.91, p=0.0308).

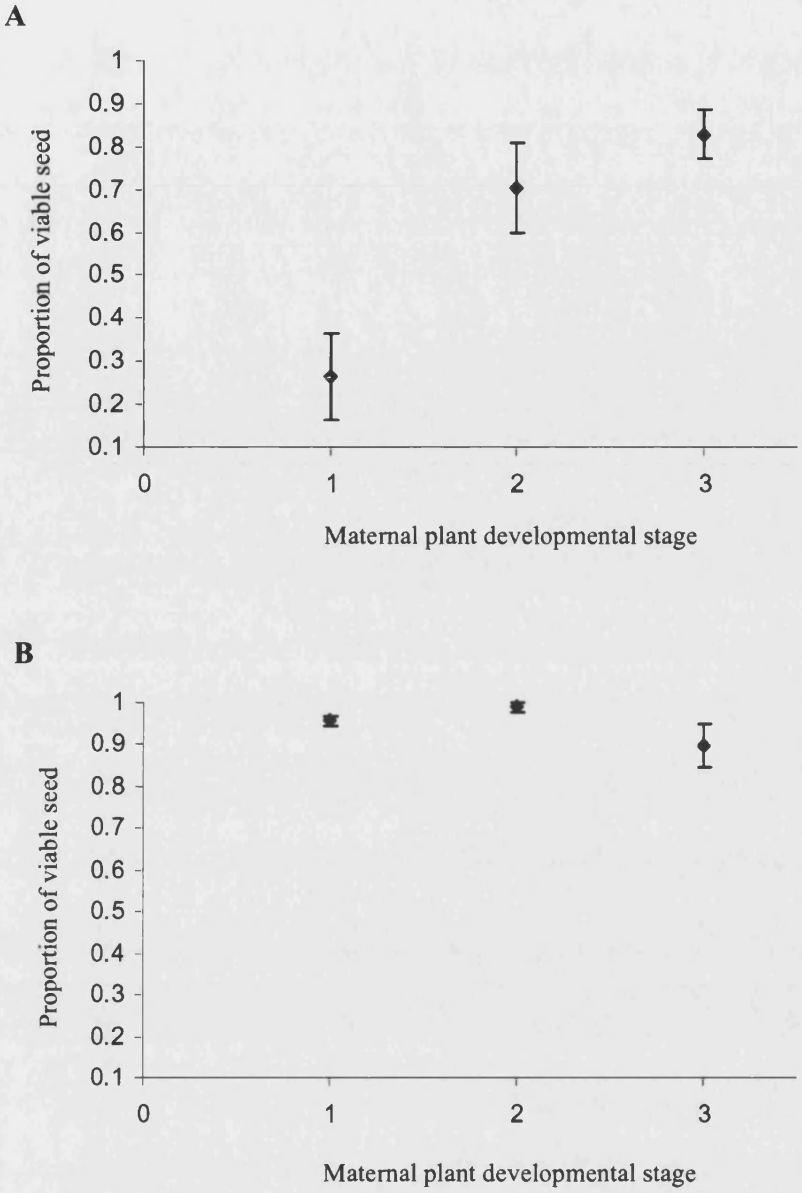


Figure 5.3

The proportion of viable seed increases with maternal age in the cross [4x X hemi*MET1a/s*] (A) but not in the reciprocal cross [hemi*MET1a/s* X 4x] (B). The average proportion of viable seed for each maternal stage is shown with a solid blue diamond. The lines represent the standard error of the mean (se).

5.2.4 The effect of removing the shoots from the maternal plant on seed mass as plant age increases

To examine if the variation in mature seed size or germination rate was a result of the experimental design, we carried out a number of control tests. There was particular concern that the removal of all the shoots of the plant, except the primary bolt, was an extremely artificial situation. Therefore to test the effect of this manipulation on seed development 4x plants were treated in one of the following 2 ways, and mature seed (from flowers that had self pollinated) was collected from the primary bolt.

1) The plants were allowed to grow naturally with no pruning
2) All the shoots bar the main bolt were removed.

In total 4 plants were used (2 for each manipulation). Seed was collected from 2 pods every 10 flowers along the primary bolt. 4x plants were used in this experiment as they produce larger seed than 2x plants and therefore it was hoped that any variation in seed size would be observed more clearly. The results are shown in Figure 5.4.

Analysis of the data using the Paired T-Test (Sokal and Rohlf, 1995) showed that there was a significant difference between the sets of seed masses from the two manipulations, indicating that removing the shoots was affecting mature seed size (T=5.34, $d.f$= 3, p=0.013). The data set from each manipulation was analysed separately with a one-way ANOVA test. If no shoots were removed (manipulation 1) there was no significant change in seed size as parental age increased (F=0.20, $d.f$=3, p=0.897). In contrast, if all the shoots were removed (manipulation 2) there was a significant increase in the size of the seed produced as the age of the plant increased (F=29.37, $d.f$=3, p<0.0001).

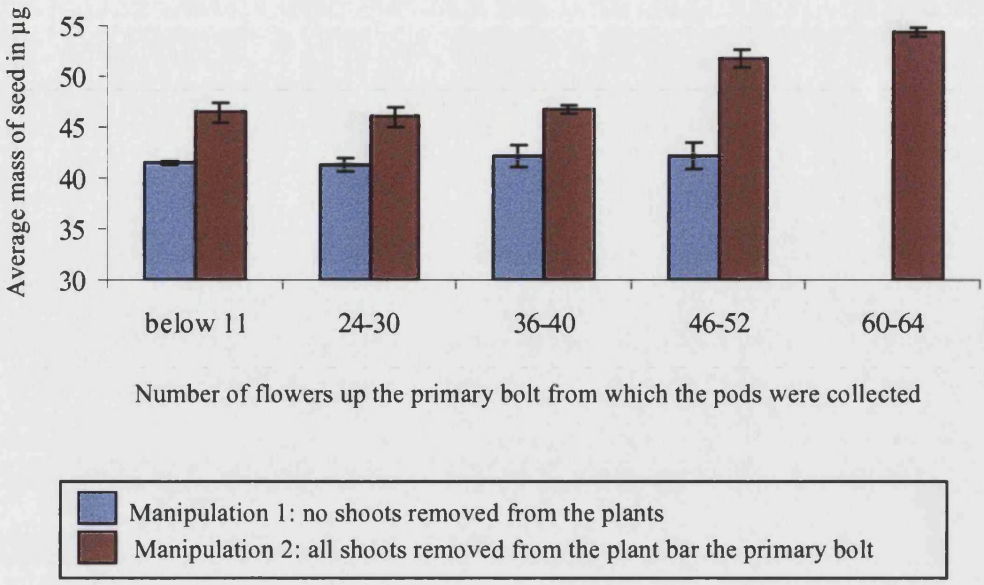


Figure 5.4

The effect of removing the shoots from a plant on seed size as the plant gets older.The vertical bars represent the s.e.m.

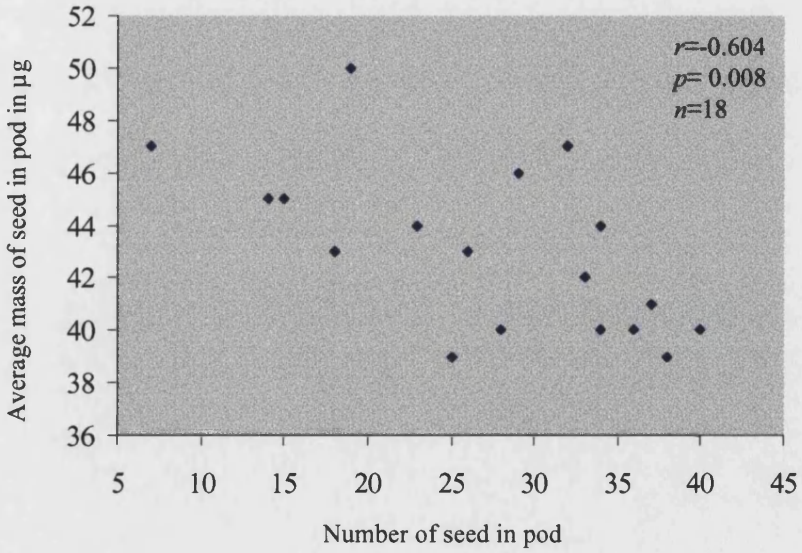


Figure 5.5

The average mass of the seed in a pod from a [4x X 4x] cross is negatively correlated to the number of seed in that pod.

5.2.5 The effect of the number of seed in a pod on average mature seed mass

The hemi*MET1a/s* plants often exhibited a number of floral homeotic defects in the later flowers. This reduced the fertility of the plant, with often only a few seeds produced per pod when stage 3 maternal plants were used in the crosses. Furthermore, the 2x and 4x plants also exhibited reduced fertility later in development. Therefore, it was proposed that the correlation observed between seed size and maternal plant age could, in part, be due to a reduced number of seed in a pod. In order to test this proposal a number of 4x flowers on the same plant were emasculated and pollinated with varying amounts of pollen from other flowers on the same plant. Each pod was tested for the total number of seeds and the average mass of the seed (Figure 5.5). Analysis of the data with the Pearson Product Moment Correlation (Sokal and Rohlf, 1995) showed there to be a significant negative correlation between the number of seed in a pod and the average mass of the seed (r= -0.604, $d.f$=16, p=0.008).

5.3 Discussion

5.3.1 Seed viability and mature mass increased with maternal plant age in a [4x X hemi*MET1a/s*] cross

In accordance with the preliminary observations made in Chapter 3, seed viability in the cross [4x X hemi*MET1a/s*] was shown to significantly increase with maternal plant age (Table 5.2, Figure 5.3) Furthermore, paternal plant age had no significant effect on seed viability. The maternal age effect on seed development was also noted with respect to seed mass. As the maternal plant got older, the average mass of the viable seed increased.

Combined, these results support the proposal that the tolerance of an imbalance of parental genomes in the endosperm of a seed increases with maternal plant age. There are numerous possible scenarios that could account for why seed with an older maternal parent could tolerate an extreme imbalance of parental genomes, and by inference an imbalance of imprinted gene expression. A conceivable cause of the seed abortion observed in the [4x X hemi*MET1a/s*] cross is starvation of the embryo due to severe under-proliferation of the PE and CE (Figure 3.7, Scott et al., 1998). An increase in the influx of nutrients to the seed could improve the chance of the seed surviving. Indeed, seed size and therefore the acquisition of resources by the progeny, has been shown to vary within a plant depending on it position (reviewed in Stephenson 1981; Lee 1988; Diggle 1995; Susko and Lovett-Doust 1998). This will be discussed in greater detail later in this Chapter.

Alternatively, the increase in seed viability in a [4x X hemi*MET1a/s*] cross could be the result of modifications to the imprinting system itself. As the maternal parent gets older the imprinting mechanism in the polar nuclei could be 'relaxed' so that normally imprinted (paternal) endosperm-promoting alleles are expressed in the developing seed. In the case of the extreme maternal excess cross [4x x hemi*MET1a/s*], the seed with an older (stage 3) 4x parent would have extra active endosperm-promoting alleles, in comparison to seed with a younger (stage 1) 4x parent. This could push the imbalance of imprinted gene expression back towards a less extreme maternal excess, therefore increasing the viability (and mature mass) of seed with an older 4x parent.

Alternatively, as the maternal plant gets older, maternally expressed endosperm-inhibiting alleles could be effectively 'turned off'. In theory this would have a similar effect on seed development in a [4x x hemi*MET1a/s*] cross as outlined above. In other words, seed with an older maternal parent could have a less extreme maternal excess, resulting in a larger average seed size and increased germination rate.

The production of larger seed by older maternal parents could theoretically confer an evolutionary advantage to the plant. According to the parental conflict theory the maternal parent regulates seed development by contributing active endosperm-limiting alleles (and inactive endosperm-promoting alleles) to the seed (Haig and Westoby, 1989, 1991; Moore and Haig, 1991; Scott et al., 1998). The maternal parent limits endosperm, and therefore seed development, as she is equally related to all her progeny and therefore maximises her 'genetic fitness' if her finite resources are spread uniformly amongst all the seed. However, it may be argued that once the plant comes towards the end of its reproductive life (for example stage 3 in the experiments) it could be an advantage to pass on more of the available nutrients, which will no longer be required by the maternal parent, to the developing seed. It has been shown that seed size can have important effects on seedling traits, including germination (Schaal, 1980; Weis, 1982; Zimmerman and Weis, 1983; Dolan, 1984) seedling size (Schaal, 1980; Hendrix and Trapp, 1992) and seedling competitive ability (Black, 1957; Houssard and Escarre, 1991). Theoretically a seedling from a large seed could have a competitive advantage, increasing its (and the parental plants) reproductive fitness.

However, these proposed mechanisms require that the maternal imprinting system is sensitive to the age of the plant. Although there could be a number of possible contributing factors it is intriguing to note that the level of genomic DNA methylation has been found to increase with plant age. The maize transposable elements *Spm* and *Mu* are more densely methylated in leaves at the top of a plant than in the first few leaves (Bennetzen et al., 1988; Banks and Fedoroff 1989; Martienssen et al.,1990; Martienssen and Baron 1994). Furthermore, young seedlings had DNA methylation levels approximately 20% lower than that of mature leaves in both tomato and

A.thaliana. (Messeguer et al., 1991; Finnegan et al., 1998). Increased DNA methylation could act in a number of different ways to modify the imprinting system.

The methylation of DNA could directly inhibit the expression of endosperm-limiting genes. Alternatively, DNA methylation could repress the expression of genes involved in the imprinting of paternally expressed alleles. A third possibility is that DNA methylation could have a less direct role with respect to the maternal age effect on the imprinting mechanisms. High levels of methylation may trigger the activity of other factors that could subsequently modify the maternal imprinting system.

The level of imprinted gene expression could also be modified by other factors than DNA methylation. For example, the expression of the imprinted murine locus $p57^{Kip2}$ decreases in the skeletal muscle and lung during aging, yet faithfully maintains both its DNA methylation and imprinting status (Park and Chung, 2001).

Although these theories are currently based on a small amount of circumstantial evidence the possibility that the imprinting system or the expression of imprinted genes could be modified during plant development is a fascinating possibility. However, before any future experiments can be proposed the results from the other experiments described in this Chapter must be discussed.

5.3.2 The viability and mature mass of seed was not affected by parental plant age in a [hemi*MET1a/s* X 4x] cross

Based on the proposal that as the age of the maternal plant increases modifications of the imprinting system occur that result in the production of larger seed, certain predictions can be made for the effect of parental plant age in the cross [hemi*MET1a/s* X 4x]. If methylation catalysed by the MET1 protein is required for the maternal age effect on seed development, and this modification is inhibited in the hemi*MET1a/s* plant, then we would predict no effect of maternal age on mature seed size (and/or germination rate) in this crosses. In contrast, if the modification is not inhibited in a hemi*MET1a/s* plant then we would predict that the seed from older maternal plants would be pushed towards a more extreme paternal excess phenotype. This could be

observed as an increase in viable mature seed size and/or an increased rate of seed abortion (i.e. towards the phenotype of a [*MET1a/s* X 4x] or a [2x X 6x] cross).

The [hemi*MET1a/s* X 4x] cross showed no significant correlation between seed viability or mature seed mass, and parental plant age (Table 5.2, Figure 5.3B). Alone this evidence supports the proposal that MET1 catalysed methylation has a role to play in the maternal age effect on seed development. However, there are a number of concerns that should be addressed.

In the original experiments described in Chapter 2, the [hemi*MET1a/s* X 4x] cross gave seed with an average germination rate of 63% (± 9.9). In contrast, overall the [hemi*MET1a/s* x 4x] crosses outlined in this chapter gave seed with an average germination rate of 94% (± 2.3). As parental age in this cross was found not to have an effect on the rate of germination this is unlikely to be the reason for variation between the 2 sets of experiments. Another possible cause of the variation could be the fact that the hemi*MET1a/s* plants were generated in different crosses. Although produced by the same method (by a [2x X *MET1a/s*] cross) the *MET1a/s* parents were different plants. Even though the plants were from the same antisense line they could have had different levels of genome methylation (Finnegan et al., 1996) and this difference could have then been inherited by the 2 sets of hemi*MET1a/s* plants.

According to the model outlined in Chapter 3 (Figure 3.16) for the role of *MET1* catalysed methylation in genomic imprinting, if the hemi*MET1a/s* plants used in the experiments described in this Chapter had less genomic hypomethylation (than the plants used in the experiments in Chapter 3) then less active endosperm-promoting alleles could be contributed to the endosperm in a [hemi*MET1a/s* X 4x] cross. This would result in a less extreme paternal excess imbalance of imprinted genes in the endosperm and therefore a greater proportion of seed may be viable.

A further clear difference between the experiments was the treatment of the plants used in the crosses. The plants used in the experiments outlined in this Chapter had all the shoots removed, while the plants in Chapter 2 were subjected to only very limited pruning. Indeed the removal of all the shoots from the primary stem was shown to

have drastic effect on seed development in [4x X 4x] crosses (discussed in detail below).

Therefore, although the observation that plant age does not significantly affect seed development in a [hemi*MET1a/s* X 4x] cross supports the hypothesis that MET1 is involved with an age dependent modification of the maternal imprinting system, there are a number of flaws with the experimental design. These should be considered in detail before any conclusions are drawn.

5.3.3 Seed development in 4x *A.thaliana* plants is affected by the removal of all the shoots except the primary bolt

Removing the shoots from a 4x plant was shown to have 2 significant effects on seed development. Firstly, the pruned plants produced seed with a mass larger than those from the untreated plants (49μg compared to 42μg, Figure 5.4). Secondly, age had a significant effect on seed size in the pruned plants, with mature mass increasing with plant age. No such correlation between plant age and seed mass was observed for the untreated plants. Both the increased seed mass and the effect of plant age in pruned 4x plants could be due to reduced competition for normally limited resources.

Plant age (or more accurately the developmental stage of the plant) has been found to affect seed development in many species. The majority of the previously reported work concerned the development of seed within inflorescences (reviewed in Stephenson 1981; Lee 1988; Diggle 1995; Susko and Lovett-Doust 1998). Typically the number and mass of seed produced has been shown to decline along the basal-to-distal axis of an inflorescence. In other words, the flowers that are produced first produce larger seed than later flowers. Intriguingly, this is the opposite trend to the situation in the pruned 4x plants.

The decrease in seed size along the inflorescence has often been viewed as a result of the competition for limited resources, as basal flowers are closer to the source of the nutrients and develop earlier, and could therefore have a spatial and temporal advantage over distal flowers with respect to the acquisition of resources (Diggle, 1995; Susko and Lovett-Doust 1998). The removal of the shoots would reduce the

competition for resources, both along the stem and in the plant as a whole, and therefore could have led to the increase in seed size in the pruned plants. Indeed previous work with *A.thaliana* showed that preventing the pollination of basal flowers in a single inflorescence led to an increase in petal, stigma and stamen size in distal flowers (Diggle, 1997). It was suggested that this was due to the reduction in competition for resources. Unfortunately there was no report on the effect on seed mass.

It should also be noted that the untreated 4x plants did not show a significant decrease in seed size along the primary stem. Although this could indicate that there is no competition for resources along the primary stem (as suggested for within individual inflorescences) the significant difference between seed mass from treated and untreated plants, suggests this is unlikely. It is possible that there is variation in seed mass, but due to the small size of *A.thaliana* seeds it was not detected by our methods.

5.3.4 Seed mass increases with maternal plant age in crosses between plants of different ages

In most of the crosses involving plants at different developmental stages seed mass was found to significantly increase with maternal plant age (Table 5.2). In isolation this observation supports the model outlined previously, that as the maternal plant ages, the imprinting system is modified resulting in the production of larger seed. However, as discussed above, the effect of plant age seed mass was only observed in 4x plants when all the shoots were removed. Since all the plants used in the crosses to test the affect of parental plant age on seed development had had all the shoots removed, this strongly suggests that the significant maternal effect on seed size observed was at least partly due to the experimental design.

Another factor that could have contributed to the maternal effect on seed mass is the reduced fertility observed in the older plants used in the crosses. The controlled pollination of 4x *A.thaliana* plants showed that as the number of seed in a pod increased the average weight of the seed decreased significantly (Figure 5.5). This in accordance with a number of studies in other plant species that have shown a similar negative correlation between seed number and mass (Agren, 1989; Melhman, 1993)

Therefore if seed number decreased as the maternal parent got older, this could have been a factor in the increase of seed mass with maternal plant age. However, it has been suggested that the relationship between seed number and seed mass is the result of competition for resources (Stephenson, 1981; Lee, 1998; Diggle, 1995). Whether such competition played a role in seed development in the pods on a pruned plant, where we have proposed that resources were plentiful is an interesting question.

5.3.5 Seed mass did not significantly increase with maternal plant age in the crosses [2x X hemi*MET1a/s*] and [hemi*MET1a/s* X 4x]

Strikingly, 2 out of the 9 crosses tested, [2x X hemi*MET1a/s*] and [hemi*MET1a/s* X 4x], did not exhibit a significant maternal age effect on seed size (Table 5.2). This was despite the fact that the experiments with the pruned and untreated 4x plants strongly suggested that the maternal age effect on seed development was due to the experimental design (Figure 5.4).

The [2x X hemi*MET1a/s*] cross produced relatively small seed (30μg) and it is possible that any variation in size was too subtle to be detected by our methods. However, the extreme maternal excess cross [4x X hemi*MET1a/s*] gave even smaller progeny (16μg) and yet showed a highly significant difference in seed mass between maternal stages 1 and 3 (p=0.0009).

The lack of maternal age effect in the [hemi*MET1a/s* X 4x] cross is intriguing. Overall, out of the 9 sets of crosses, this cross produced the largest seed (47μg ± 0.97), which suggests that any increase in mature mass would be detected with ease. The hypomethylation of the hemi*MET1a/s* parent may have had a role to play in perhaps diminishing the effect of maternal age on seed size. However, the other 2 sets of crosses with the hemi*MET1a/s* plants as the maternal parent, ([hemi*MET1a/s* X hemi*MET1a/s*] and [hemi*MET1a/s* X 2x]), both exhibited a significant maternal age effect on mature seed mass. Another possible explanation could be that the seed from a [hemi*MET1a/s* X 4x] cross had reached the maximum possible mature mass for *A.thaliana*. In this scenario, factors either in the surrounding maternal tissue or in the seed, could act to limit the influx of any more nutrients, constraining the size of the

seed. If a seed grew over a particular size this could be detrimental, both to itself and its siblings, even with no competition for nutrients, due to space limitations in the pod.

5.3.6 Seed mass increases with paternal age in a [2x X 4x] cross

Of the 9 sets of crosses tested 8 showed no significant effect of paternal age on mature seed mass. This is to be expected if the increase in seed mass was solely due to the pruning of the nutritive maternal plant. However, the [2x X 4x] cross did show a significant increase in seed mass as the paternal 4x plant aged. Although there is the possibility that this result is a statistical error (perhaps due to too small a sample size) there are a number of possible reasons why a paternal effect on seed mass may only have been observed in a [2x X 4x] cross.

The [2x X 4x] cross produced relatively large seed compared to most of the other crosses tested, so that changes in seed mass may have been observed more clearly by our methods. If, as suggested above, the seed from a [hemi*MET1a/s* X 4x] cross had reached their maximum size then constraints laid down by the maternal parent could have masked any affect of paternal age on seed mass. As the [2x X 4x] seed did increase in mass as the maternal plant got older, and therefore had not theoretically reached this 'wall of maximum size', this may have allowed the observation of the perhaps more subtle paternal effect on seed size.

It is difficult to comprehend how paternal age could affect seed development, apart from some alteration in the imprinting system, as the pollen contributes little to the developing seed apart from the genetic material. Furthermore, it is not easy to predict an evolutionary advantage for older paternal parents to produce larger seed. According to the parental-conflict theory of imprinting the paternal parent reaches maximum genetic fitness if nutrients go preferentially to his progeny, regardless of the effect on the development of unrelated siblings (Haig and Westoby, 1989,1991; Moore and Haig, 1991). Therefore the paternal parent would reach maximum fitness if he always strove to produce large seed, despite whether he is at the beginning or end of his reproductive life, as (at least in out-crossing species), there is no drain on paternal plant resources.

An interesting possibility is that a paternal age effect on seed mass could be an adaptation of the imprinting system which has evolved in pre-dominantly self-pollinating species. As outlined in Section 1.3.4 it has been proposed that *A.thaliana* inherited an imprinting system from an outcrossing ancestor (Stebbins, 1974; Scott et al., 1998) and that this system has become attenuated, allowing the tolerance of an imbalance of imprinted genes in the endosperm, as observed in reciprocal crosses between 2x and 4x plants. Could the system have also adapted to compensate for the fact that the maternal and paternal parents are, in 98% of cases, the same plant? (Abbott and Gomes, 1989). In other words, could the paternal imprinting system have evolved to be 'relaxed' in younger plants, therefore preventing excessive growth of the seed at the expense to the parental plant? As the plant reaches the end of its reproductive life the 'relaxation' of the paternal imprinting system may be reduced, so that more active endosperm-promoting alleles (or fewer active endosperm-inhibiting alleles) could be contributed to the endosperm, resulting in an increased mature seed mass.

Such hypotheses are highly speculative and are based on only a small amount of experimental data. However, they do emphasize the fact that little is known about the evolution of imprinting in plants and highlight an important point that the imprinting system may not be 'black and white' and could be modified in different situations (e.g. as the plant gets older).

5.3.7 The effect of plant age on seed development

The ultimate objective of the experiments described in this chapter was to determine if seed development was effected by the age of the plants in a cross. The work was based on the preliminary observation (in Chapter 3) that in crosses between hemi*MET1a/s* and 4x plants the proportion of viable seed increased with maternal age. This result was replicated in the cross [4x X hemi*MET1a/s*]. However, whether the positive correlation between seed mass and maternal plant age that resulted from the removal of all shoots from the plant except the primary stem plays a role in the increased seed viability is difficult to deduce. Even if this is a contributing factor in these experiments it does not explain the original observations of the effect of plant

age on germination rate in a [4x X hemi*MET1a/s*] cross, as these plants were subjected to limited pruning. Therefore it still remains a possibility that the age of plants in a cross could affect the tolerance of a seed to an imbalance of imprinted genes in the endosperm.

The effect of plant age on mature seed mass was also difficult to analyse due to the consequence of removing all the shoots on seed development. However, it was shown that the mass of seed on the primary stem of a 4x plant did not show significant variation with respect to the age of the plant if the shoots were not removed. This suggests that plant age may not directly affect seed mass. In order to clarify these contradictory results the stage experiments should be repeated with plants that have not had the shoots removed. The plant could be developmentally staged as before, by counting the number of flowers along the primary stem.

In conclusion, the effect of parental age on seed development in crosses between 4x and hemi*MET1a/s* plants still remains unclear. Further study of this possible phenomenon may not only give us a greater insight into the process of genomic imprinting and seed development as a whole, but could also provide us with tools to study and manipulate seed growth.

Chapter 6

The expression pattern of *MET1* and *DRM2* during floral, gamete and seed development

6.1 Introduction

6.1.1 Background

The objective of the work described in this Chapter was to define further the role of DNA methylation in the parent-of-origin effects, and by inference imprinting, in *A.thaliana*. The complementary phenotype of seed from reciprocal crosses between wild type and *MET1a/s* plants (described in Chapter 3) supported the model that MET1 plays a global role in the parent-of-origin effect. However, the timing of this imprinting-associated methylation is not known and how it is subsequently propagated in the developing seed was unclear.

6.1.2 The establishment of imprinting-associated methylation

Determining the time point at which imprinting-associated methylation occurs could help pin point when imprints are set in plants, regardless of whether DNA methylation is the primary imprinting mark in this system. As many flowering plants are hermaphrodites, imprints themselves must be set following the separation of the precursors of the male and females gametes. This could occur at any stage between stamen and carpel initiation, and fertilization (Figure 3.15).

As discussed in Chapter 3, the results of crosses between hemi*MET1a/s* and wild-type plants provided some evidence about the timing of imprinting-associated methylation. The progeny of these crosses developed according to the methylation status of the parents, regardless of whether they inherited the *MET1a/s* transgene. One possible explanation for this is that at least some element of the parent-specific methylation is set prior to the first nuclear division of meiosis, before the segregation of the *MET1a/s* transgene. Alternatively, the gametes themselves may not express MET1, but inherit the protein from the diploid spore mother cells. A third possibility is that it is the

epigenetic status of the DNA entering the imprinting process that is important for the setting of imprinting. If this is the case, it can be envisaged that the establishment of imprinting might be due to parental specific maintenance of methylation as opposed to parent-specific *de novo* methylation. One way to help begin to distinguish between these diverse models is to determine the expression pattern of *MET1*.

Further evidence that indicates the developmental period at which MET1 is required for the imprinting-associated DNA methylation, comes from a number of studies that suggest that the 35S promoter, which drives the expression of the *MET1a/s* transgene, is inactive in the developing male gametophytes (Wilkinson et al., 1997; Custers et al., 1999; Table 3.3). If the 35S promoter is inactive then the expression of MET1 is unlikely to be affected in these tissues in *MET1a/s* plants and therefore would be available to establish or propagate imprints. This suggests that the action of the MET1 protein with respect to imprinting-associated methylation, which is attenuated in *MET1a/s* plants, does not occur during this stage of male gametophyte development.

However, there is evidence that the 35S promoter is active in all 4 whorls of the organ primordia in the floral meristem (Mandel, 1992; Kritez et al., 1996; Liljegren et al., 1999) Therefore the MET1 protein could be important in the establishment of imprinting-associated methylation during floral organ differentiation, but it was not known if *MET1* was actually expressed within these cells in wild type flowers.

6.1.3 The propagation of imprinting-associated methylation

Although the evidence presented in this Thesis suggests that MET1 protein is required to establish and/or propagate imprinting-associated methylation prior to gametogenesis, the possibility remained that MET1 could still have an important role in propagating imprinting methylation patterns during gametogenesis and/or seed development. Indeed, the parent-of-origin effects in seed from reciprocal crosses between *MET1a/s* and wild type plants suggests that the imprinting-associated methylation patterns are maintained on chromosomes inherited from the wild type parent. Furthermore, there is evidence that the 35S promoter (and therefore the *MET1a/s* transgene) is not active in the embryo or endosperm of a developing seed, until it reaches heart-torpedo stage (F.Berger personal communication, Boisnard-

Lorig et al., 2001). Therefore, even in seed that contain the *MET1a/s* transgene, the MET1 protein could be produced and have a role replicating imprinting-associated methylation. However, the role of MET1 in propagating imprinting-associated methylation was difficult to determine, as there was no information available on the wild type expression profile of *MET1* during gametogenesis or seed development.

Therefore in order to further define the role of DNA methylation in the parent-of-origin effects we performed a detailed study of the wild-type expression profile of *MET1* in floral and reproductive tissues and developing seed. This expression profile was intended not only to allow us to distinguish between the different models for the action of MET1 in establishing and/or propagating imprinting-associated methylation, but also provide us a tool with which to study the role of MET1 in plant development as a whole.

6.1.4 The potential role of other DNA methyltransferases in the parent-of-origin effects

Although clear evidence was available that MET1 plays a major role in the parent-of-origin effects, and by inference imprinting, any of the other 8 putative DNA methyltransferases in the completed *A.thaliana* genome could also have a role in establishing and/or propagating imprinting-associated methylation. However, as with *MET1*, little was known about the exact expression profile of these genes during flower and seed development. Ideally to comprehensively study the role of DNA methyltransferases in the parent-of-origin effects obtaining a detailed expression profile of all these putative enzymes would have been desirable. However, due to time limitations the decision was made to study the expression of only one additional gene, the *DRM2* gene. This locus was given preference as it was predicted to encode a *de novo* DNA methyltransferase (Cao et al., 2000). As MET1 is attributed with having a predominantly maintenance methyltransferase activity (Finnegan and Kovac, 2000) we envisaged that another enzyme might be required to actually establish the imprinting-associated methylation. In this scenario, MET1 could then have a role in maintaining the sex-specific methylation patterns (perhaps set by the action of DRM2) after DNA replication.

6.2 Results

6.2.1.1 Construction of *MET1* and *DRM2* GUS expression constructs

To further define the role of DNA methylation in the parent-of-origin effects in *A.thaliana* we proposed to study the wild type expression pattern of *MET1* and *DRM2* during floral development, gametogenesis and seed development. For this purpose we constructed and analysed transgenic plants carrying GUS reporter genes driven by each respective promoter, which required the following steps.

1) The amplification of the promoter (*MET1*) or promoter gene sequence (*DRM2*) from genomic DNA.
2) The fusion of the promoter sequence with the GUS reporter gene.
3) The building of a binary vector with the promoter GUS transgene incorporated to allow the introduction of the transgene into plant genomic DNA via *A.tumefaciens* transformation.
4) The production of plant lines that expressed the GUS reporter gene from the required promoter by *A.tumefaciens* mediated transformation.

6.2.1.2 The design of primers to amplify the *MET1* promoter sequence

To amplify the *MET1* promoter from genomic DNA, primers were designed against sequences upstream of the *MET1* coding sequence. The Genbank database was used to identity a 1.9kb length of sequence lying upstream of the *MET1* ATG start codon (Accession number AF139372). A BLAST search with the AF139372 sequence identified a TAC clone (Accession number AB016872) that contained additional upstream sequence. Primers were designed to amplify a 2508bp fragment of the *MET1* promoter. The reverse primer MET1PB2 amplified the sequence from the nucleotide immediately upstream of the ATG start codon. To allow for directional cloning of the fragment into the BJ60 vector restriction site linkers were incorporated into both the forward and the reverse primers.

Table 6.1

The nucleotide sequence of the primers used to amplify the *MET1* promoter

Primer name	Primer sequence (5' to 3')	Restriction site linker[1]
MET1PF	AAAGGTACCCTCTGTAGATCGTGCATTATC G	Kpn1
MET1PB2	AAGGATCCTTTCAAAATCCCTAGTTTCAAAATCAAAATTACC	BamHI

[1]The restriction sites of the linkers are underlined

6.2.1.3 The design of primers to amplify the *DRM2* promoter-gene sequence

The primers to amplify the *DRM2* promoter from genomic DNA were designed to anneal to the *DRM2* genomic sequence deposited in the Genbank database (Accession number AL163792). The primers amplified a 3008bp fragment incorporating 1.9kb of promoter sequence and 1.1kb of coding sequence. The coding sequence was included, as this has been found to stabilise transgene expression (J.Coles, personal communication). Unfortunately, this information was not known prior to the design of the MET1 expression construct. The primers were also designed to ensure that the coding sequence remained in frame with the coding sequence of the GUS gene when ligated into the BJ60 plasmid.

Table 6.2

The nucleotide sequence of the primers used to amplify the *DRM2* promoter-gene sequence

Primer name	Primer sequence (5' to 3')	Restriction site linker[1]
DRM2F	AAGGTACCATACGCATCAGGTCAAACTATGCG	Kpn1
DRM2B	AAGGATCCTACTGCTGCTGGCAGTTTCTTAGC	BamHI

[1]The restriction sites of linkers are underlined

6.2.1.4 The Construction of the *MET1*promoterGUS Recombinant Binary Plasmid (*MET1::GUS*) and the *DRM2*promoter/geneGUS Recombinant Binary Plasmid (*DRM2::GUS*)

The *MET1*promoter PCR fragment was ligated into the pGEMT vector (Figure 6.1 A). The MET1PGEMT plasmid was then digested with *Kpn1* and *BamH1* and the 2.5 kb *MET1*promoter fragment isolated. The BJ60 vector, containing the GUS gene, was also digested with *Kpn1* and *BamH1*. The *MET1*promoter fragment was cloned into the BJ60 vector to form the *MET1*BJ60 recombinant plasmid (Figure 6.1 B). The position of the restriction sites in the multicloning region of BJ60 ensured that the MET1promoter fragment was orientated in the same direction as the GUS gene (5' to 3'). The *MET1*BJ60 plasmid was subsequently digested with the *Not1* restriction enzyme to release the cassette of approximately 4.5kb, containing the *MET1*promoter, the NLSGUS gene and the nos3' terminator. The BJ40 binary vector was also digested with *Not1* and the *MET1::GUS* cassette ligated in to form the recombinant *MET1::GUS* plasmid (Figure 6.1C). The *DRM2::GUS* binary plasmid was made as described above for the *MET1::GUS* binary plasmid.

6.2.1.5 The production of transgenic plants carrying the GUS expression constructs

The GUS expression constructs were transformed into *A.tumefaciens* and introduced into *A.thaliana* ecotype Col, via the floral dipping method. The efficiency of transformation varied from 0.8% for the *MET1::GUS* experiment to 0.08% for the *DRM2::GUS*construct (see Table 6.3).

Table 6.3

Transformation efficiency for the *MET1::GUS* and *DRM2::GUS*

Construct used to transform *A.thaliana*	Number of primary transformants (T1)[1]	Efficiency of transformation (Number of T1/25000)[2]
MET1::GUS	200	0.8%
DRM2::GUS	21	0.084%

[1] Number of kanamycin resistant seedlings obtained from 8 pots of plants that had been subjected to the floral dipping process

[2] Based on 25 000 seed weighing 0.5g

20 random primary transformants (T1) were selected for each construct and were allowed to self-fertilize to produce secondary transformant (T2) seed. The T2 plants were then tested for the number of insertions of the transgene. Those with single insertions were chosen for further characterization. Unfortunately, due to time limitations, only a few transgenic lines were analysed for each construct. For *MET1::GUS* lines 4 and 10 were studied in detail, with some preliminary work conducted with line 16. For *DRM2::GUS*, lines 8 and 13 were studied.

6.2.2 The expression profile of *MET1*

To clarify the role of the MET1 enzyme in the establishment and propagation of imprinting-associated methylation the wild type expression pattern of the *MET1* gene was examined by studying GUS activity in transgenic plant lines expressing the GUS reporter gene driven by the *MET1* promoter.

6.2.2.1 GUS staining in the floral and reproductive tissues of *MET1::GUS* plants

MET1::GUS flowers showed a high level of staining in the anthers and ovaries (Figure 6.2 A-C). Intense staining was also observed in the vascular tissue of the stem, filaments and ovaries. Preliminary experiments also indicated that there was GUS activity in the floral meristem of *MET1::GUS* flowers (data not shown).

Pollen from *MET1::GUS* plants showed a high level of GUS activity (Figure 6.3) Figure 6.3 A shows a representative anther from a line hemizygous for the

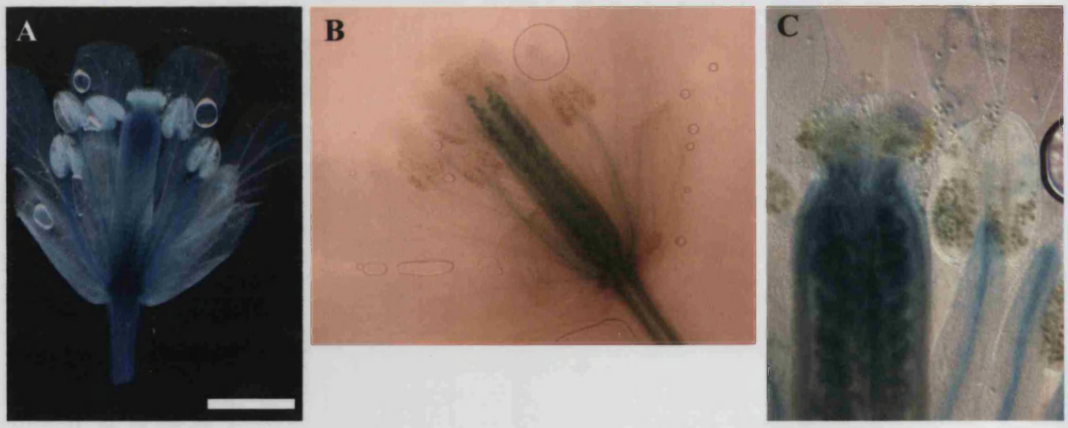


Figure 6.2

Histochemical staining of *MET1::GUS* flowers. (A) image captured using the SPOT camera and sample lighted from below (B) image captured with a digital camera and sample illuminated from above (C) shows an increased magnification of the stigma and anthers. This image was captured with the spot camera and was illuminated from below. In all 3 images note the intense staining of the vascular tissue of the funiculus, the anther filaments and the ovary wall. The pollen and ovules also stain for GUS activity. Scale bar, 1mm.

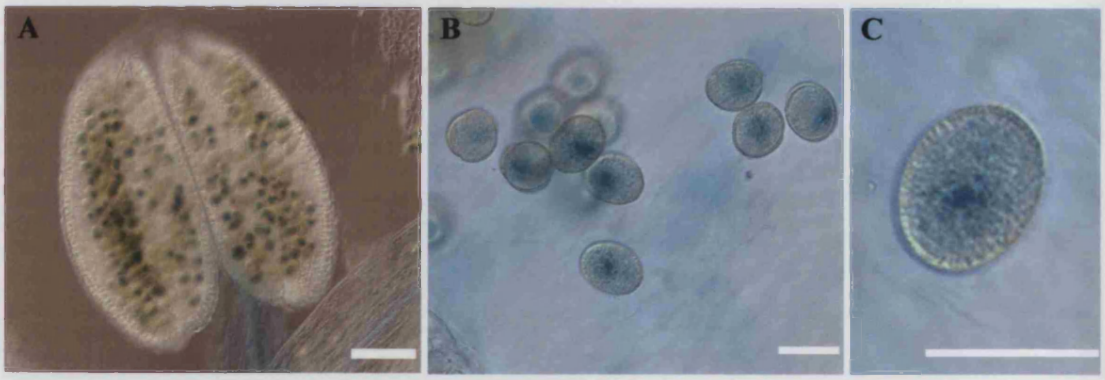


Figure 6.3

Histochemical staining of *MET1::GUS* pollen. (A) shows an anther from a plant hemizygous for the *MET1::GUS* transgene. 50% of the pollen stained for GUS activity. Within the pollen the stain was localized to a central region (B and C). Scale bar (A) 0.05mm, (B) and (C) 10um.

MET1::GUS transgene. The surrounding maternal tissues of the anther were unstained whilst approximately 50% of the pollen showed GUS activity. The expression of the GUS transgene was more intense within a particular region of the pollen grain, however due to the limitations of the technique it was not possible to determine if this region coincided with the generative or sperm cells.

Ovules from *MET1::GUS* flowers showed reproducible patterns of GUS activity, with intense staining in the chalazal pole, the central cell and the vascular tissues of the funiculus (Figure 6.4 A and B). The integuments showed no activity above background levels at the micropylar or chalazal poles. GUS activity was present however in the integuments in the region in between the 2 poles. In approximately 90% of the ovules 3 to 4 intensely stained regions were noted in the central region of the embryo sac and at the micropylar pole (Figure 6.4 A). The remaining ovules exhibited fewer or no such intensely stained regions (Figure 6.4 B).

6.2.2.2 GUS staining in developing seed from *MET1::GUS* plants

To gather evidence for the role of MET1 in the propagation of imprinting-associated methylation after fertilization the temporal and spatial expression of the MET1 gene during seed development was examined by studying GUS activity in seed from the *MET1::GUS* lines. Preliminary experiments showed that GUS activity in the *MET1::GUS* lines was very high in the integuments. This did not allow us to observe any potential GUS activity in the embryo or endosperm. For this reason seed were also examined from crosses between a maternal 2xC24 parent and a paternal *MET1::GUS* plant, as the integuments are maternally derived tissues. Where possible plants hemizygous for the *MET1::GUS* transgene were used in these crosses to provide an internal control. If half the seed from these crosses showed no GUS activity this would suggest that any GUS staining observed in the seed was due to the activity of the *MET1::GUS* transgene and not due to endogenous activity.

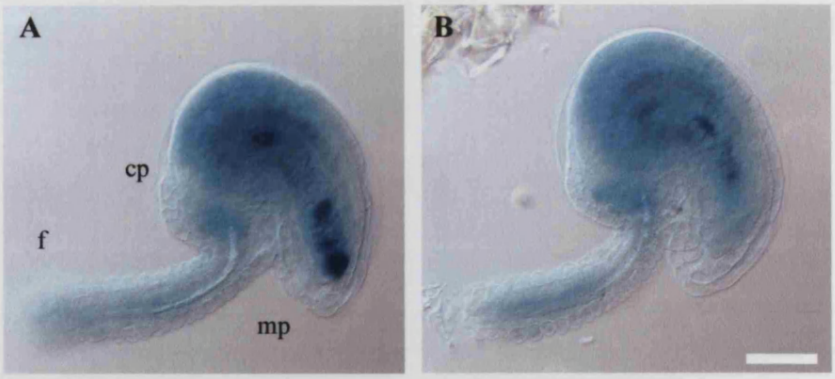


Figure 6.4

Histochemical staining of *MET::GUS* ovules. (A) shows the characteristic staining pattern of approximately 90% of *MET1::GUS* ovules, with staining in the funiculus (f), the central cell and a region at the chalazal pole (cp). Staining was also observed in dense structures within the central cell and at the micropylar pole (mp). (B) some seed showed fewer, or none, of these densely stained regions. Scale bar, 0.05mm.

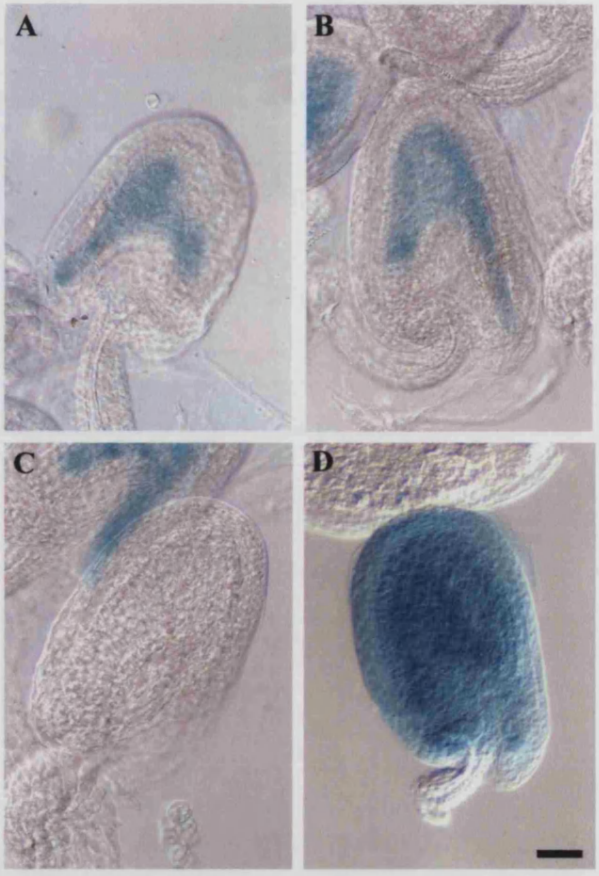


Figure 6.5

Histochemical staining of 1DAP seed carrying a *MET1::GUS* transgene. When the *MET1::GUS* transgene was paternally inherited GUS staining was observed throughout the central cell with both line 10 (A) and line 4 (B). (C) shows one of the 50% of ovules that did not stain when a hemizygous *MET1::GUS* plant (in this case line 4) was used as the paternal parent. (D) shows a typical staining pattern if the *MET1::GUS* transgene was used as the maternal parent (in this case with line 4). The extensive staining of the integuments masked any potential staining of the embryo or endosperm. Scale bar, 0.05mm

6.2.2.2.1 Seed that inherited a paternally derived *MET1::GUS* transgene showed extensive GUS activity in the embryo sac, which by 3DAP became confined to the embryo and a region of the chalazal pole

1DAP seed that inherited a paternally derived *MET1::GUS* transgene showed extensive staining throughout the entire embryo sac (Figure 6.5 A and B). By 2 DAP these seed showed GUS activity in the globular embryo (Figure 6.6 A to C, E and F). However, the staining pattern of the other tissues at 2DAP depended on the transgenic line. Where the *MET1::GUS* line 10 was used, staining was confined to the embryo in approximately 50% of seed (Figure 6.6 A). In 25% of the seed diffuse staining was also observed throughout the centre of the embryo sac, as well as more concentrated staining within the embryo (Figure 6.6 B). The remaining 25% of these seed showed distinct staining of the embryo and a small region within the chalazal pole (Figure 6.6 C). In contrast, when the *MET1::GUS* line 4 was used as the paternal parent the 2DAP seed showed staining throughout the embryo sac, as well as within the embryo (Figure 6.6 E and F). The staining of the central embryo sac varied from light to dense (compare 6.6 E and F respectively). In the latter case a region at the chalazal pole was often intensely stained.

By 3DAP, in the majority of seed that inherited a paternally derived *MET1::GUS* transgene GUS activity was confined to the embryo (Figure 6.7). However, in a small proportion of seed (approximately 5%) additional GUS staining was observed at the chalazal pole (Figure 6.7 B, E and G-H). In the example shown (Figure 6.7) the GUS activity was localized to a single cell, which was closely associated with the maternal CPT. As described above, seed that inherited a maternally derived *MET1::GUS* transgene showed intense staining of the integuments at all described stages (e.g. Figure 6.5 D).

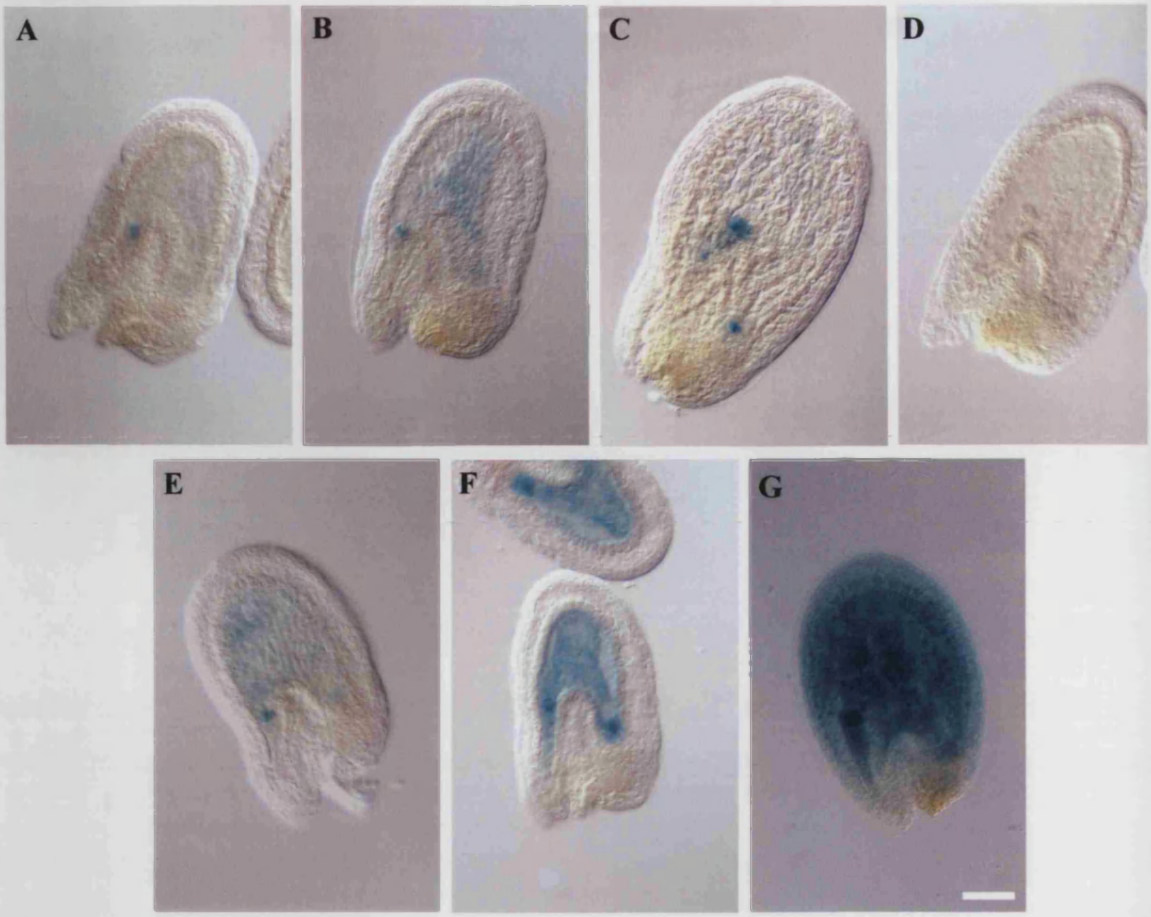


Figure 6.6

Histochemical staining of *MET1::GUS* seed 2DAP. The different expression patterns observed when the *MET1::GUS* transgene was paternally inherited are shown in A to D (line 10) and E and F (line 4).With line 10, most seed (approximately 80%) showed GUS staining only in the embryo, although a small proportion of seed (about 15%) still stained for GUS activity in the central cell of the seed (B).A few seed (approximately 5%) also showed specific GUS staining within a region of the chalazal pole (C). If a plant hemizygous for the transgene was used, 50% of the seed showed no detectable GUS activity (D). A less specific pattern of GUS staining was observed if line 4 was used as the paternal *MET1::GUS* transgene donor (E and F). Staining was present throughout the central cell of all the seed 2DAP, although denser staining was seen in the globular embryo. The intensity of the staining varied (compare E and F) between seed. Note in (F) the staining at the chalazal pole despite the absence of activity in the surrounding tissues of the central cell. When the *MET1::GUS* transgene was inherited via the maternal parent there was extensive staining of the maternally derived integuments (G). Dark staining of the globular embryo could often be observed. Scale bar, 0.05mm.

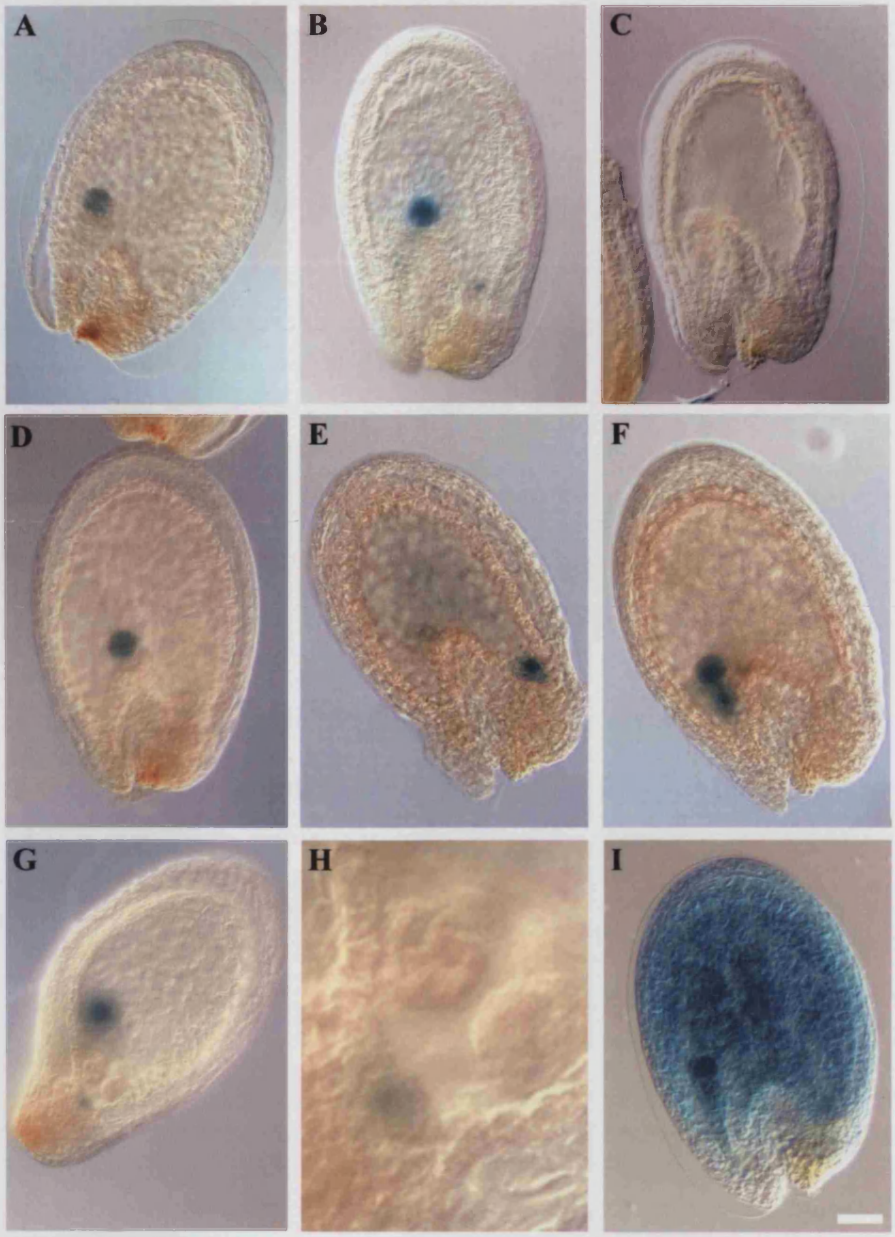


Figure 6.7

Histochemical staining of *MET1::GUS* seed 3DAP. The different staining patterns observed when the *MET1::GUS* transgene was paternally inherited are shown in A to C (for line 10) and D to H (for line 4). (A) most line 10 seed showed GUS activity only in the globular embryo, although a small proportion (approximately 5%) also stained in a small region at the chalazal pole(B). When the paternal plant used was hemizygous for the *MET::GUS* transgene approximately half of the seed showed no detectable GUS staining (C). (D) most seed that inherited a paternal line 4 *MET::GUS* trangene also only showed staining in the embryo (D) although in a few cases (less than 10%) staining was also observed at the chalazal pole (E, G and H) or the suspensor (F). (H) is a magnified section of the chalazal pole of the seed shown in (G) and shows GUS staining localised to a single cell that is closely associated with CPT. (I) shows a typical pattern of staining that was observed when the *MET1::GUS* transgene (in this case line 4) was inherited from the maternal parent. Note the extensive staining of the integuments.Scale bar, 0.05mm.

6.2.2.2.2 In seed that inherited a paternal *MET1::GUS* transgene, GUS activity was confined to the embryo 5DAP

5DAP seed, with a paternally inherited *MET1::GUS* transgene, showed positive staining for GUS activity only within the heart shaped embryo (Figure 6.8 A to C). In contrast, if the *MET1::GUS* plants were used as the maternal parent dense staining was observed in the integuments (Figure 6.8 E and F). Interestingly, the staining was of a greater intensity within the region of the embryo and the chalazal pole.

By 8DAP, in seed that inherited a paternally derived *MET1::GUS* transgene the GUS staining was still predominately localized to the torpedo shaped embryo (Figure 6.9) Within the embryo a central region that ran through the cotyledons and the stem of the embryo exhibited denser staining than the rest of the embryonic tissue. In some cases diffuse staining was also observed in the tissues directly surrounding the embryo (Figure 6.9 A).

6.2.2.2.3 In later stage embryos that inherited a *MET1::GUS* transgene GUS activity became localized to specific regions of the embryo

It was difficult to test the activity of the GUS transgene in seed at later stages of development (after approximately 10DAP) due to the reduced penetrance of the histochemical reagent. Therefore, in order to test if the *MET1::GUS* transgene remained active in later stage embryos, embryos were carefully removed and stained from seed 12DAP and mature seed (approximately 21DAP).

The 12DAP embryos showed a branched staining pattern (Figure 6.10 A and B). Within the stem of the embryo the GUS staining was localized to a central core region, possibly the vascular tissue. The cotyledons showed a conserved forking pattern of staining around a centrally stained region. Again this probably represents staining of the vascular tissues. Embryos from mature seed exhibited a similar pattern of GUS activity to that of the 12DAP stage embryos (Figure 6.10 D). However, the intensity of the staining was greatly reduced, particularly within the cotyledons.

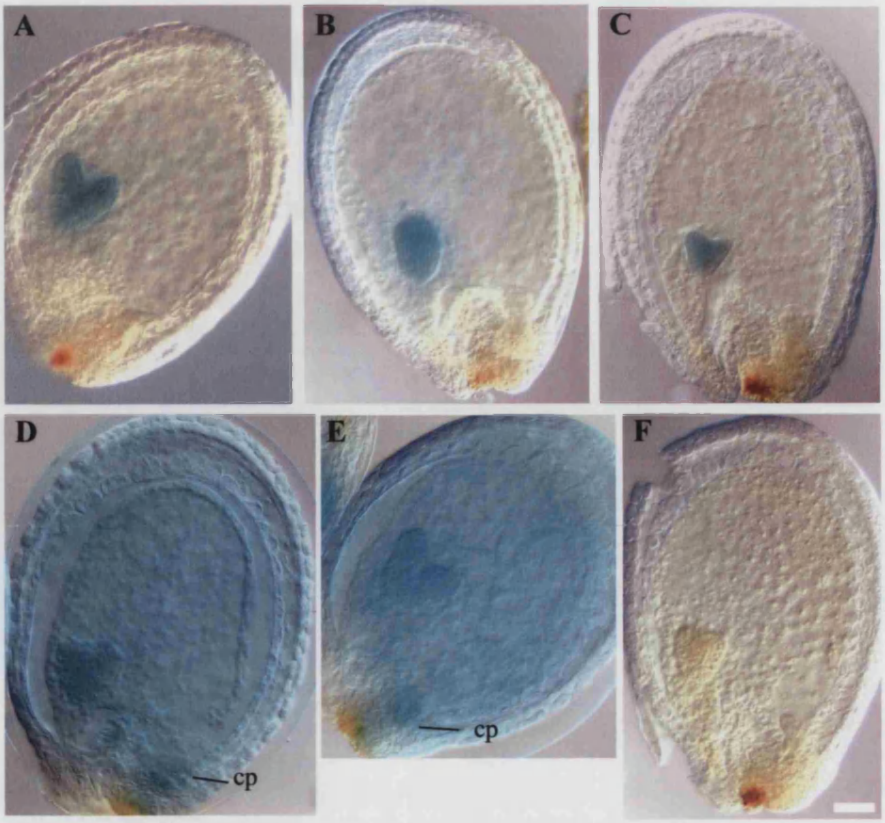


Figure 6.8

Histochemical staining of *MET1::GUS* seed 5DAP. When the transgene was paternally inherited GUS activity was localized to the embryo (A) line 4, (B) line 10, (C) line 16. In contrast, when the transgene was inherited from the maternal parent GUS staining was observed in all of the maternally derived integuments (D and E). However, denser staining could be observed in the embryo and at the chalazal pole (cp). (F) shows one of the 50% of seed that showed no detectable GUS activity when the *MET1::GUS* lines used as the paternal parent were hemiyzgous for the transgene. Scale bar, 0.05mm.

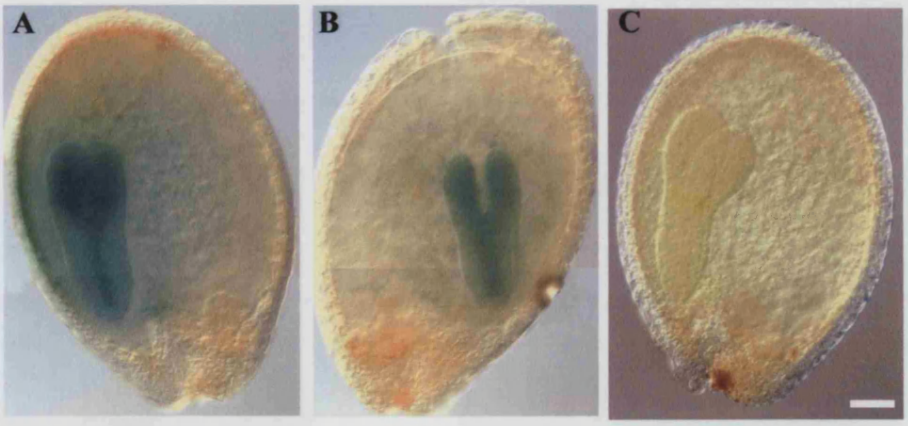


Figure 6.9

The staining pattern of seed with a paternally inherited *MET1::GUS* transgene 8DAP. The GUS staining was localized to the torpedo embryo (A and B, lines 4 and 10 respectively). Within the embryo the GUS staining was more intense in the central region of the embryonic root and cotyledons. When a plant hemizygous for the transgene was used, 50% of the seed exhibited no detectable GUS activity (C). Scale bar, 0.05mm.

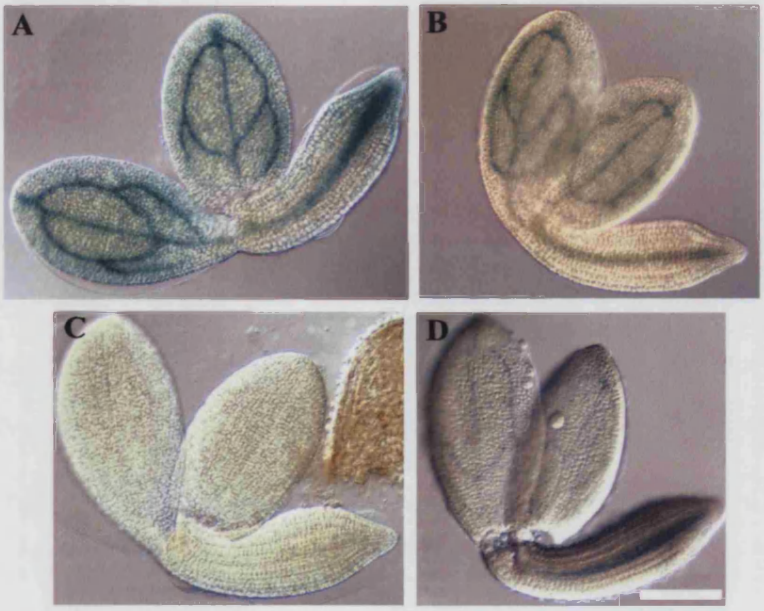


Figure 6.10

Histochemical staining of *MET1::GUS* embryos from seeds 12DAP and 21DAP (mature seed). (A and B) show the branched staining pattern of embryos from 12 DAP seed for lines 16 and 10 respectively. In each case the transgene was inherited from the paternal parent. When the paternal parent was hemizygous for the transgene 50% of the embryos exhibited no detectable GUS activity (C). (D) shows an embryo from a seed 21DAP which inherited the transgene (line 16) from the paternal parent. Note the expression pattern is similar but far weaker than in embryos observed 12DAP. Scale bar, 0.05mm.

6.2.3 The Expression profile of DRM2

To determine whether the putative *de novo* DNA methyltransferase DRM2 might have a role in the establishment and/or propagation of imprinting-associated methylation the wild type expression pattern of *DRM2* was studied in flowers, gametes and developing seed using *DRM2::GUS* transgenic lines.

6.2.3.1 GUS staining in floral and reproductive tissues of *DRM2::GUS* plants

To study the wild type expression pattern of the *DRM2* gene during floral and gamete development, flowers from *DRM2::GUS* transgenic lines were histochemically stained, and the tissues examined for GUS activity. The *DRM2::GUS* lines showed intense staining in the stem, the ovary walls and in the vascular tissue of the petals, sepals and stamen filaments (Figure 6.11 A to D).

Approximately 10% of the pollen from a plant hemizygous for the *DRM2::GUS* transgene showed detectable GUS activity (Figure 6.12 A to D). No staining was observed in any other tissue of the anther. The pollen that did exhibit GUS activity showed diffuse staining throughout the central region of the pollen, with more intense GUS activity in the nucleate structures.

Ovules from the *DRM2::GUS* lines showed interesting patterns of GUS activity (Figure 6.13 A and B). An intense region of staining was observed within the central cell at the micropylar pole of the ovule. Flecks of stain were also noted in region of the central cell closest to the micropylar pole. No GUS activity was noted in the chalazal region of the central cell, or in the integuments or funiculus.

Figure 6.11

Histochemical staining of *DRM2::GUS* flowers. (A) image captured with a SPOT camera and the sample illuminated from below. (B) image captured with a digital camera and the sample lit from above. Note the intense staining in the vascular tissue of the petals, sepals and funiculus. (C) shows an increased magnification of the stigma, anthers and part of the ovary. (D) shows the intense staining of the vascular tissue of the petals (p) and the sepals (s). The vascular tissue of the anther filaments (f) showed little if no GUS activity. Scale bar, 1mm

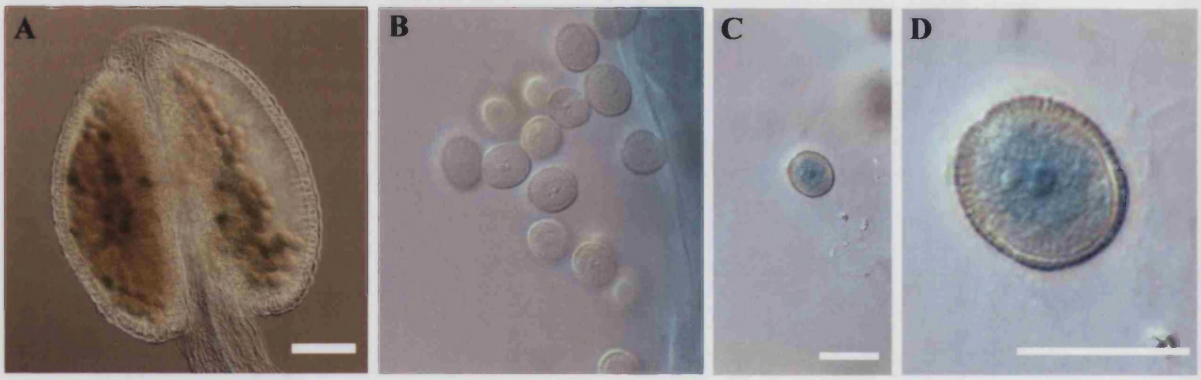


Figure 6.12

Histochemical staining of *DRM2::GUS* pollen. (A) shows an anther from a plant hemizygous for the transgene. Most of the pollen showed no detectable GUS activity (B), although a small proportion showed staining in the central region (C and D) with denser staining in the nucleate regions. Scale bar (A) 0.05mm, (C) and (D) 10um.

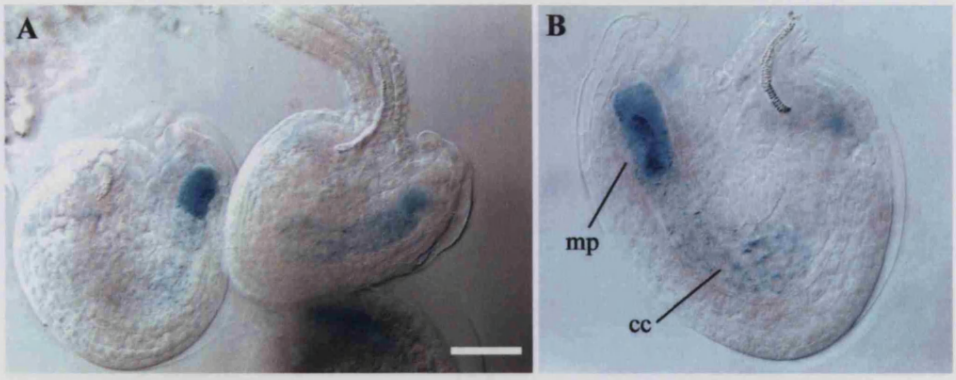


Figure 6.13

Histochemical staining of *DRM2::GUS* ovules. (A) shows the staining pattern observed in most ovules (left) and the less intense pattern exhibited by approximately 10% of the ovules (right). (B) shows a more detailed example. Note the intense staining at the micropylar pole (mp) and the flecks of stain within the central cell (cc). Scale bar, 0.05mm.

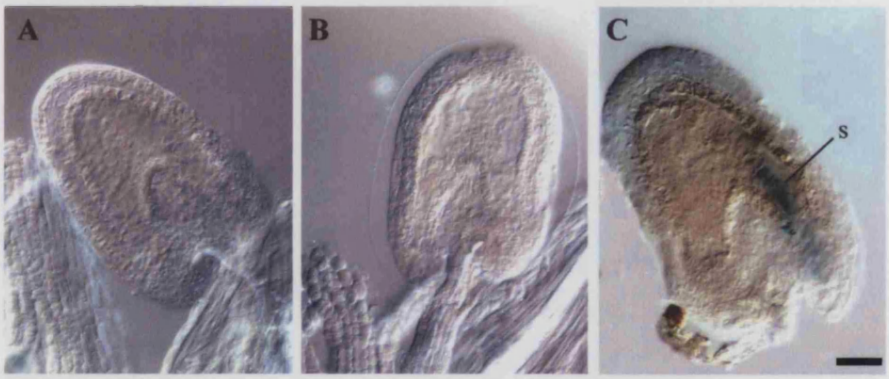


Figure 6.14

Histochemical staining of *DRM2::GUS* seed 1DAP. No detectable GUS actvity was observed if the transgene was inherited from the paternal parent (A and B, lines 8 and 13 respectively). In contrast, if the *DRM2::GUS* transgene was inherited from the maternal parent (in this case line 8) distinct staining was observed in the suspensor (s) (C). Scale bar, 0.05mm

6.2.3.2 GUS staining in developing seed from *DRM2::GUS* lines

To determine whether DRM2 plays a role in the propagation of imprinting-associated methylation after fertilization we determined the expression pattern of DRM2 during seed development. For this purpose seeds from transgenic *DRM2::GUS* lines at different DAP were tested for GUS activity. Preliminary experiments showed the staining of seed with a maternal *DRM2::GUS* parent to be highly variable, with the seed coat often showing dense patches of staining that on occasions obscured the view of the embryo or endosperm. For this reason seed were also examined from crosses between a maternal 2xC24 parent and a paternal *DRM2::GUS* plant..

6.2.3.2.1 The *DRM2::GUS* transgene was active in the suspensor 1DAP when maternally inherited, but was inactive until 3DAP when inherited from the paternal parent

In early stage seed (1DAP to 2DAP) no GUS activity was observed if the *DRM2::GUS* transgene was inherited from the paternal parent (Figure 6.14 A-B and 6.15 A-B). In contrast, if the transgene was maternally inherited localized GUS activity within the suspensor was noted 1DAP (Figure 6.14 C). This activity became more widespread in 2DAP seed with staining observed in the suspensor, embryo, a region of tissue at the chalazal pole and patches of the integuments (Figure 6.15 C).

By 3DAP seed showed a high level of GUS activity in the suspensor regardless of whether the *DRM2::GUS* transgene was inherited from the maternal or paternal parent (Figure 6.16). Seed that inherited a maternal transgene also showed staining in patches of the integuments and in the tissues and integuments surrounding the suspensor, in addition to the more intense GUS activity in the suspensor (Figure 6.16 C-E). In contrast, the majority of seed with a paternally derived *DRM2::GUS* transgene only showed GUS activity in the suspensor (Figure 6.16 A and D). However, when line 13 was used as the paternal parent 10% of the seed exhibited additional staining in the embryo (Figure 6.16 B).

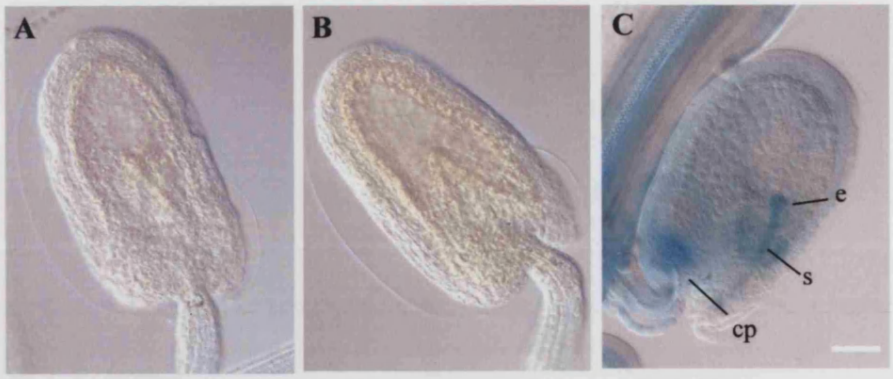


Figure 6.15
Histochemical staining of *DRM2::GUS* seed 2DAP. When the transgene was inherited from the paternal parent no detectable GUS activity was observed in the seed (A and B) lines 8 and 13 respectively. However, when the transgene was inherited from the maternal parent staining for GUS activity was observed in the embryo (e), the suspensor (s) and a region at the chalazal pole (cp) (C). Scale bar, 0.05mm.

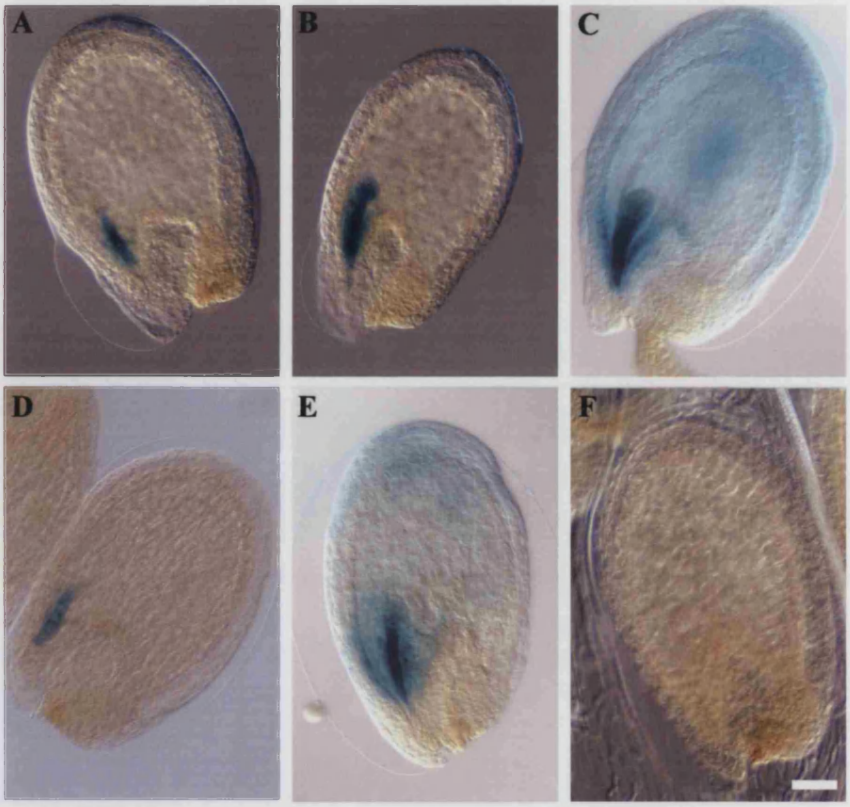


Figure 6.16
Histochemical staining of *DRM2::GUS* seed 3DAP. The expression of the GUS transgene was dependent of the line used and whether the transgene was inherited through the maternal or paternal parent. When line 13 was used as the paternal parent most seed showed staining in only the suspensor (A) although some seed also exhibited GUS activity in the embryo (B). When the GUS transgene was maternally inherited from line 13, staining was observed in the suspensor, embryo and surrounding tissues (C). When line 8 was used as the paternal parent, staining was only observed in the suspensor (D). In contrast, when line 8 was used as the maternal parent GUS activity was noted in the suspensor, embryo and surrounding tissues (E): When the transgenic line (in this case line 8) used as the paternal parent was hemizygous for the transgene 50% of the seed showed no detectable GUS activity (F). Scale bar, 0.05mm.

6.2.3.2.2 GUS activity was observed in the embryo and suspensor in *DRM2::GUS* seed 4 to 5 DAP

Seed with a maternally derived *DRM2::GUS* transgene showed high GUS activity in the suspensor, as well as less intense staining in the embryo and the tissues surrounding the suspensor (Figure 6.17 B and D). By 5DAP, the level of GUS activity in the embryo had increased in these seed (Figure 6.18 B) and staining was still observed in the suspensor and surrounding tissues.

Seed that inherited the *DRM2::GUS* transgene from the paternal parent also showed GUS activity within the embryo and suspensor, but the time point at which the embryo activity was observed differed between the transgenic lines. When line 13 was used as the paternal parent embryo GUS activity was noted in 4DAP seed (Figure 6.17 A). However, when line 8 was used in the same crosses embryo GUS activity was not observed until 5DAP.

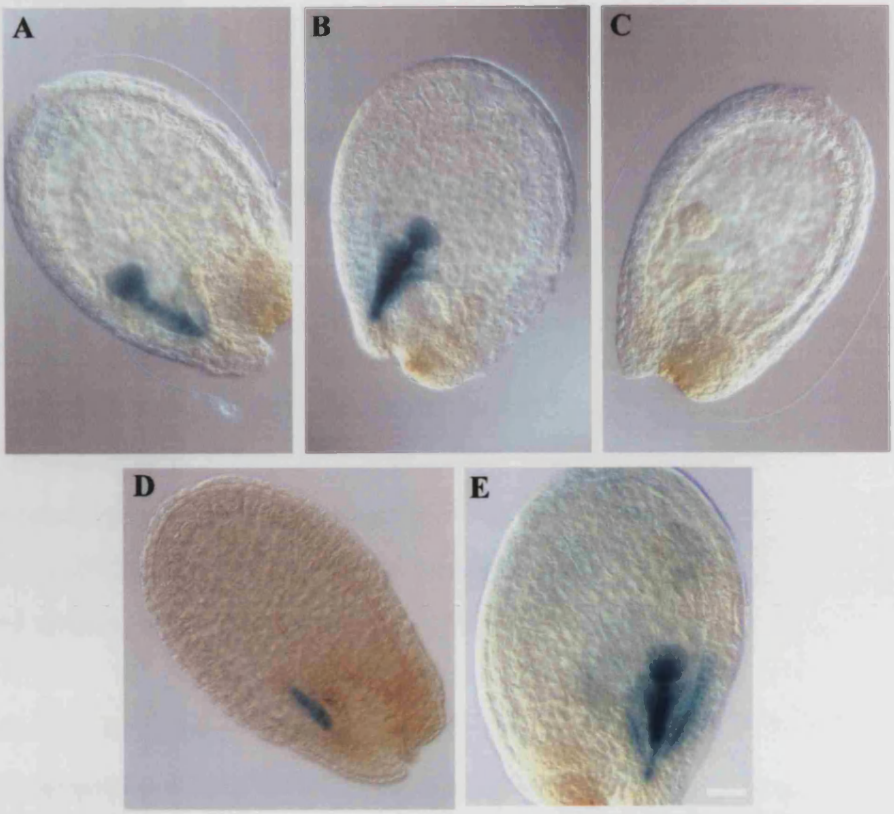


Figure 6.17

Histochemical staining of *DRM2::GUS* seed 4DAP. As with the previous Figure the pattern of GUS staining varied with the transgenic line used. When line 13 was used as the paternal parent GUS staining was localized to the embryo and suspensor (A).When the line 13 was used as the maternal parent additional staining was also observed in the surrounding tissues (B). When the *DRM2::GUS* plant used as the paternal parent was hemizygous for the transgene, 50% of the seed showed no detectable staining (C). In contrast, when line 8 was used as the paternal parent GUS activity was localized only to the suspensor (D). When line 8 however was used as the maternal parent staining was observed in the embryo, suspensor and surrounding tissue (E). Scale bar, 0.05mm.

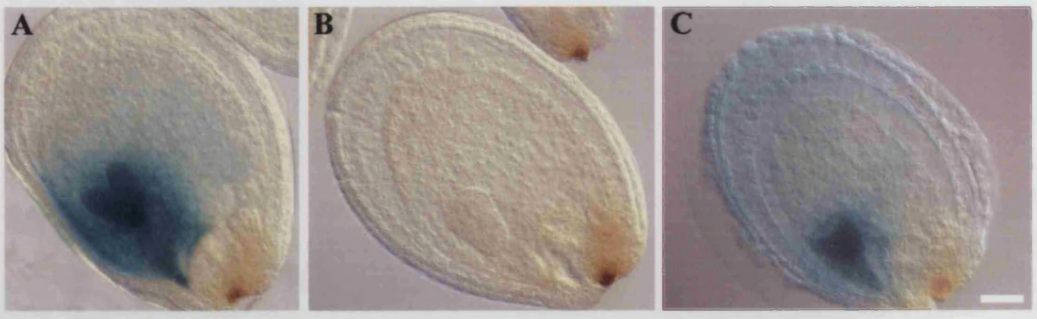


Figure 6.18

Histochemical staining of *DRM2::GUS* line8 seed 5DAP. When the *DRM2::GUS* transgene was paternally inherited the seed showed extensive staining in the embryo, the suspensor and the surrounding tissues (A). When the paternal plant used in these crosses was hemizygous for the transgene 50% of the seed showed no GUS activity (B).When the transgene was maternally inherited a similiar pattern of GUS activity was observed as in (A) except that there was additional staining of the maternally derived integuments (C).Scale bar, 0.05mm

6.2.3.2.3 *DRM2::GUS* seed showed a high level of GUS activity in the embryo at later stages of seed development (8DAP to seed maturity)

8DAP seed with a paternal *DRM2::GUS* parent exhibited staining in the embryo and the surrounding tissues (Figure 6.19 A and B). However, the level of staining was highly variable and often faint. To test whether this variability was due to the limited penentrance of the histochemical reagent, embryos were removed from the rest of the seed prior to the staining process. The isolated embryos showed intense staining in all the tissues (Figure 6.19 D). The seed from which the embryos had been extracted also showed localized staining within the suspensor (Figure 6.19 E).

To determine whether the intense staining observed in embryos from *DRM2::GUS* seed 8DAP was maintained during the later stages of development embryos from seed 10DAP and mature seed were tested for their GUS activity. 10DAP embryos had intense staining throughout all the tissues (Figure 6.20 A). In contrast, in embryos extracted from mature seed the staining was localized to the central regions of the cotyledons and the stem (Figure 6.20 B). No staining was observed in the outer tissues of the cotyledons or in the tissue that connects the cotyledons to the 'main stem' of the embryo. The tissue at the dorsal tip of the embryonic root also showed no GUS activity.

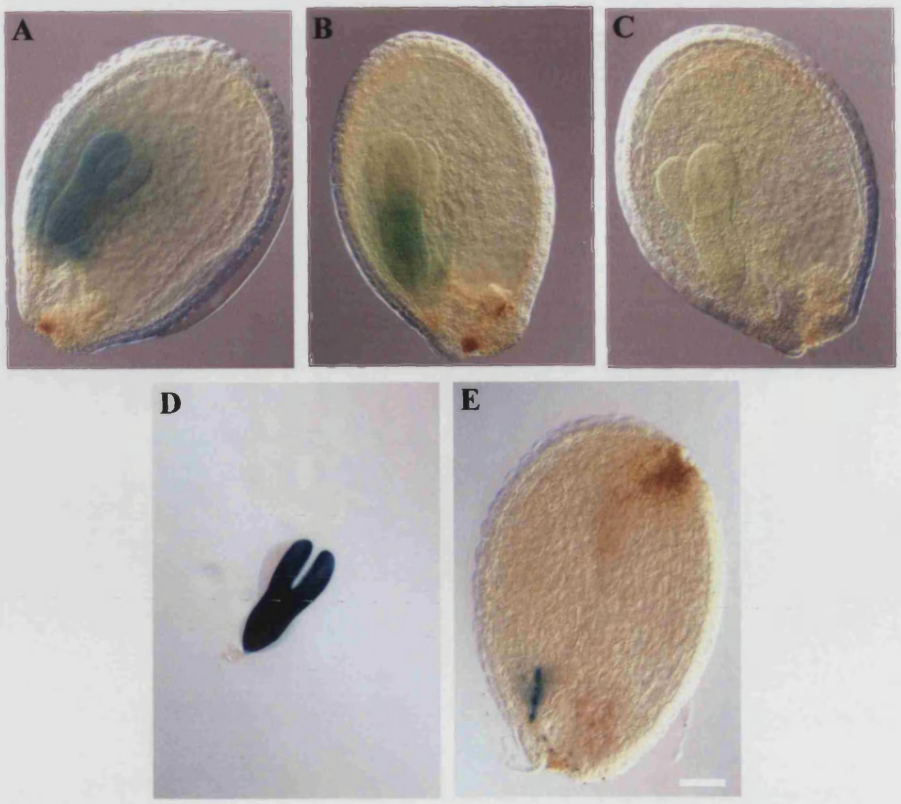


Figure 6.19

Histochemical staining of seed with a paternally inherited *DRM2::GUS* transgene 8DAP. All the examples in this figure are from crosses with line 8, but are also representative of the crosses with line 13. The seed showed staining of the embryo which could vary from complete staining (A) to partial staining (B). When the plant used was hemizygous for the transgene 50% of the seed showed no detectable GUS activity (C). When the embryo was removed from the seed prior to the histochemical treatment the entire embryo showed intense GUS staining (D). The suspensor that remained attached to the seed coat also stained positive for GUS activity (E). Scale bar, 0.05mm.

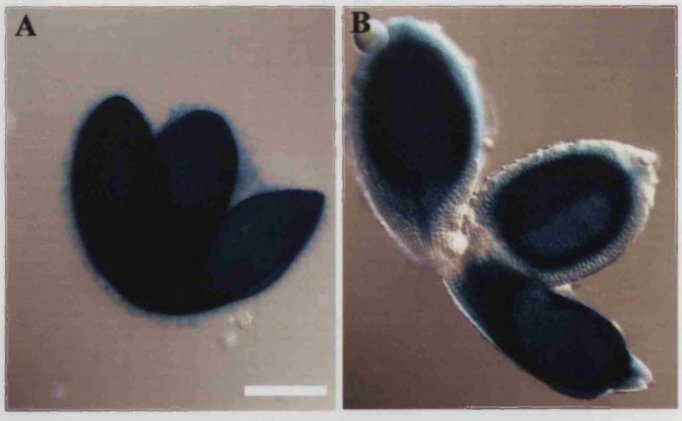


Figure 6.20

Histochemical staining of *DRM2::GUS* embryos from 10DAP (A) and mature (B) seed. The intense staining of the embryo observed in 8DAP seed was still present in 10DAP stage embryos. By seed maturity the staining had become more localized to the central regions of the embryonic tissue. The examples shown are embryos that inherited the transgene from a paternal line 8 parent. Similar results were obtained when line 13 was used in the same experiments (data not shown). Scale bar, 0.05mm.

6.3 Discussion

DNA methylation has long been attributed with a role in mammalian genomic imprinting, yet the exact role of this epigenetic modification in the parent-of-origin effects, and by inference genomic imprinting, in *A.thaliana* remains elusive. The aim of this of the worked described in this Chapter was to further define this role by studying the wild type expression profile of the DNA methyltransferases, *MET1* and *DRM2*, during periods of development when imprinting-associated methylation is likely to be established or propagated.

6.3.1 The setting of imprints in *A.thaliana*

In mammals there is strong evidence that DNA methylation acts as the primary imprinting mark and that these imprints are set during gametogenesis (reviewed in Mann et al., 2001; Arney et al., 2001). In contrast, whether methylation acts as the imprinting mark in plants is currently unknown. Nevertheless, determining the point at which imprinting-associated methylation is established in plants could provide important information on the setting of imprints in plants.

As outlined in Chapter 3, evidence for the period when MET1 catalyzed imprinting-associated methylation might be established came from crosses between *hemiMET1a/s* and wild type plants. The seed from such crosses developed according to the methylation status of the parents, regardless of whether they inherited the transgene. However, interpretation of these results was difficult due to the lack of information on the wild type expression of *MET1*.

One possible explanation is that the gametes themselves do not express MET1, but may inherit MET1 protein or even mRNA from the diploid spore mother cells. However, the *MET1::GUS* expression lines reported in this Chapter showed a high level of GUS activity in both ovules and pollen, providing evidence that does not support the hypothesis. Although the possibility remains that the GUS transcript was inherited from the parental diploid tissues this appears unlikely as, particularly in the case of the pollen, the surrounding tissue rarely displayed detectable GUS activity.

An alternative explanation proposed to account for the uniform phenotype of seed from the *hemiMET1a/s* crosses was that at least some component of the imprinting-associated methylation is set prior to the first meiotic division of gametogenesis. Consistent with this proposal, there is evidence that the 35S promoter, which drives the *MET1a/s* transgene, is not active at least during male gametogenesis. Therefore the participation of MET1 in imprinting, which is clearly diminished in *MET1a/s* plants, is unlikely to occur during this period of development. Moreover, there is evidence that the 35S promoter is active in all 4 whorls of the organ primordial (Mandel, 1992; Kritez *et al.,* 1996; Liljegren *et al.,*1999) which lends support to the idea that MET1 could act to establish and/or propagate imprinting-associated methylation during this time and it could be this action that is attenuated in *MET1a/s* plants.

The preliminary data indicated that the floral meristems of *MET1::GUS* plants positively stained for GUS activity. This suggests that MET1 could act to establish MET1 imprinting-associated methylation during this period. MET1 directed methylation could act as the primary imprinting mark. Alternatively MET1 could add methylation onto alleles that had already been epigenetically marked for imprinting. This could serve to reinforce the imprinted state ensuring it is stably inherited through multiple rounds of cell division. Indeed, in the transcriptional gene silencing (TGS) of transgenes, DNA methylation was shown not to be a prerequisite for silencing, although it appeared to be required for the maintenance of the established epigenetic state (Dieguez et al., 1998).

However, the proposals discussed above are based on the assumption that the setting of imprints will involve the addition of sex-specific methylation to alleles subject to imprinting, either as the mark itself, or as part of a more complex epigenetic modification. An intriguing alternative is that the setting of imprints or imprinting-associated methylation could rely on the methylation status of the incoming somatic DNA.

In this scenario, the sex-specific loss of methylation on incoming DNA could lead to the establishment of imprinting-associated methylation. Another possibility is that the methylation patterns on DNA could lead to the recruitment of sex-specifically

expressed chromatin components. In this hypothesis, the DNA of *MET1a/s* or *hemiMET1a/s* plants would lack the DNA methylation patterns that normally provide the substrate for imprinting, so imprints or imprinting associated methylation cannot be established. Here, the setting of imprints or imprinting-associated methylation could occur at any time when the precursors of the female and male gametes become separated, but the MET1 dependent methylation patterns, whose loss results in the parent-of-origin effects on seed development, must be set prior to gametogenesis. Indeed, theoretically imprinting-associated methylation could be established at any time point after fertilization (Figure 3.15).

6.3.2 The propagation of imprinting-associated methylation during gametogenesis

If imprinting-associated methylation is established prior to gametogenesis it follows that these epigenetic patterns will be then propagated during gamete development. Both the *MET1::GUS* and the *DRM2::GUS* lines showed high levels of GUS activity in the pollen and the ovules, providing circumstantial evidence that either enzyme may be involved in the replication of such sex-specific methylation. This is supported further by the more intense localization of staining to possible nucleate structures within the gametes, where the replication and methylation of DNA should occur. Indeed the mammalian DNA methyltransferases DNMT1, DNMT3a and DNMT3b have all been localized to the nucleus (Leonhardt et al., 1992, Margot et al., 2001).

Interestingly, *MET1::GUS* and *DRM2::GUS* showed different patterns of expression in the gametes. *MET1::GUS* was expressed throughout most of the ovule and pollen, although appearing most intense in the nucleate structures. Furthermore, *MET1::GUS* plants hemizygous for the transgene exhibited no detectable GUS activity in 50% of the pollen. This suggested that the staining observed in the pollen was due to transgene activity and not caused by endogenous expression.

In contrast, *DRM2::GUS* was specifically expressed in a region at the micropylar pole of the ovule in a region that contains the egg and central cell. Also, when a plant hemizygous for the *DRM2::GUS* transgene was tested only 10% of the pollen stained positive for GUS activity. One possible explanation is that the staining in these few

pollen grains was the result of endogenous GUS activity. Alternatively, the anthers examined from the *DRM2::GUS* plants may have contained pollen at slightly different stages of development, and therefore only the grains that were at the correct development stage (and carried the GUS transgene) would have stained for GUS activity, if the transgene is expressed for a short window of time. Further detailed study of the expression pattern of the *DRM2::GUS* trangene throughout pollen development would allow the testing of this hypothesis.

In summary, the analysis of GUS activity in ovules and pollen from *MET1::GUS* and *DRM2::GUS* transgenic lines strongly suggests that both enzymes are expressed for a period during gamete development. Although the exact profile of expression remains to be determined, this preliminary evidence indicates that both MET1 and DRM2 could propagate imprinting-associated methylation during gametogenesis. The inferred high expression of *MET1*, and to a lesser extent *DRM2*, in the ovules and pollen suggests that these enzymes could have vital roles in gamete development as a whole.

6.3.3 The propagation of imprints and imprinting-associated methylation during seed development

A core feature of genomic imprinting is the differential expression of alleles depending on their parental origin. This necessitates the propagation of imprints that were set prior to fertilization in the developing offspring. Indeed, the complementary phenotypes of seed from reciprocal crosses between *MET1a/s* and wild type plants indicated that imprinting-associated methylation was maintained on the chromosomes inherited from the wild type parent. As there is evidence that the 35S promoter, which drives the *MET1a/s* transgene, is not active in either the endosperm or embryo until the embryo reaches heart-torpedo stage, MET1 could have a role in propagating this methylation. It is also conceivable that other methyltransferases, such as DRM2, may have a similar role. Study of the expression of *MET1::GUS* and *DRM2::GUS* during seed development provided exciting information on the potential role of these enzymes, not only with respect to imprinting but also with regards to other phenomena such as paternal genome silencing (Vielle-Calzada et al., 2000). Due to

the complex nature of the expression profiles of *MET1* and *DRM2* each will be considered separately.

6.3.3.1 The *MET1::GUS* transgene was expressed at high levels in 1DAP seed, regardless of whether the transgene was inherited from the maternal or paternal parent

Seed with a paternal *MET1::GUS* parent (and a wild type maternal parent) examined 1DAP showed a high level of GUS activity throughout the central cell. This expression became predominantly localized to the embryo by 2DAP (discussed in greater detail in the next Section). When the transgenic plant was used as the maternal plant there was very high GUS activity in the integuments, which masked any staining of the embryo or the central cell. Thus most of the discussion will focus on seeds that were generated using a paternally supplied transgene.

The high level of GUS activity in these young seeds is evidence to support MET1 having a role in the propagation of imprinting-associated methylation during early seed development. Indeed the burst of transgene expression from the paternal genome (and presumably also from the maternal genome) suggests that MET1 may have a very important role in seed development at this stage. One such role could be to take part in a process similar to the genome wide demethylation and remethylation of DNA, which occurs in the mammalian embryo prior to implantation (reviewed in Arney et al 2001; Mann et al., 2000). However, the observation that the progeny of hypomethylated *hemiMET1a/s* plants, which fail to inherit the transgene, do not regain wild type levels of methylation is evidence against the existence of such a process in plants (Finnegan et al., 1998).

An alternative action of MET1 could be to further methylate the genome at this early developmental stage, perhaps with the methylated DNA acting as a prerequisite or template. One function for this process could be the silencing of incoming transposable elements thereby protecting the integrity of the genome (Finnegan and McElroy, 1994). Another function of the burst of MET1 activity could be to participate in the silencing of paternally inherited loci during early seed development.

The silencing of most, if not all, of the paternal genome during early seed development was recently proposed by Vielle-Calzada et al., (2000). In a study of 19 transposant lines, which expressed GUS in the embryo and/or endosperm, no detectable GUS activity was observed in early seed (up to 80 hours after pollination), when the transposon was inherited from the paternal parent. As the transposants represented insertions spread throughout the genome the authors inferred that early embryo and endosperm development is controlled by maternal genome alone. MET1 could act to silence the paternally inherited genome, either directly or indirectly by reinforcing the silent state.

However, the expression of GUS from the paternally inherited *MET1::GUS* transgene also indicates that the entire paternal genome is unlikely to be silenced during early seed development. This is in accordance with another report that showed a paternally inherited *AtRPS5A::GUS* transgene (which expresses GUS driven by the ribosomal-subunit protein 5A promoter) exhibits detectable GUS activity in the embryo as early as the 2 cell stage (Weijers et al.,2001). Unfortunately, due to time limitations we were unable to test whether the paternally inherited *MET1::GUS* transgene is active earlier than 1DAP.

The above data provides a clear demonstration that seed with a paternal *MET1::GUS* parent (and a wild type maternal parent) display extensive GUS activity throughout the central cell 1DAP. Assuming the *MET1::GUS* expression pattern accurately reflects that of the *MET1* gene this data shows that MET1 is expressed in the tissues that form the endosperm and could play a role in propagating imprinting-associated methylation during this key developmental stage.

6.3.3.2 The expression of the paternally inherited *MET1::GUS* transgene localizes to the embryo after 2 to 3DAP, with limited activity in the CE

The expression of the paternally inherited *MET1::GUS* transgene became increasingly localized to the embryo after 2 to 4 DAP. The exact time point at which this localization occurred varied between the different transgenic lines. This variance in expression could be due to the different point of insertion of the transgene and further lines should be tested to define the expression profile. Nevertheless, by the late

globular-early heart stage of development *MET1::GUS* expression was undetectable in the endosperm, suggesting that MET1 is unlikely to participate in the imprinting-associated methylation after this time. However, in two *MET1::GUS* transgenic lines a few seed at this stage showed staining within a very small region at the chalazal pole. In a one example this GUS activity was localized to a single chalazal endosperm cell that was closely associated with the maternal CPT. This suggests that *MET1* is expressed briefly within the developing chalazal endosperm and therefore might have a role in propagating imprinting-associated methylation in this tissue at a this stage. Indeed it has been reported that the *Dnmt1o* oocyte specific variant of the mammalian DNA methyltransferase DMNT1 is required for the maintenance of imprinting methylation during a single round of cell division in the developing embryo (Howell et al., 2001).

6.3.3.3 The paternally inherited *MET1::GUS* transgene was expressed only in the embryo after 3DAP, but might also be expressed in the CE if inherited from the maternal parent

As discussed above the expression of the paternally inherited *MET1::GUS* transgene was confined to the embryo 4DAP (when the embryo had reached heart stage). Alone this observation suggests that *MET1* is not expressed in any part of the endosperm after the embryo has reached early-heart, and therefore is unlikely to function in propagating imprinting-associated methylation in these tissues. However, it should be noted that in seed with a maternally inherited *MET1::GUS* transgene a region at the chalazal pole was observed to stain more intensely than other surrounding tissues. Although the staining of the maternally derived integuments prevented a conclusive assessment of GUS expression within the chalazal endosperm the intriguing possibility remains that the *MET1::GUS* transgene, and perhaps endogenous *MET1*, could be expressed in a parent-of-origin specific manner. If *MET1* is expressed in the chalazal endosperm the *MET1* gene may not only be imprinted, but could also play a role in maintaining this state by propagating imprinting-associated methylation.

6.3.3.4 A role for MET1 in the establishment and propagation of imprinting-associated methylation

The data presented in this Chapter is not sufficient to assign a precise role to MET1 in the parent-of-origin effects. However, the construction and analysis of the *MET1::GUS* plants has allowed the potential role(s)of this enzyme in establishing and propagating imprinted-associated methylation to be narrowed down. We propose that the action of MET1, which is diminished in *MET1a/s* plants resulting in the parent-of-origin effects, occurs prior to gametogenesis. We further propose that MET1 may also have an important role in propagating imprinting-associated methylation throughout gametogenesis and initial seed development.

6.3.3.5 The potential role of DRM2 in the propagation of imprinting-associated methylation during seed development

Although *DRM2* is predicted to encode an enzyme with predominantely *de novo* DNA methyltransferase activity this does not preclude DRM2 a role in propagating imprinting-associated methylation during seed development. Indeed, DRM2 could have maintenance DNA methyltransferase activity under certain conditions. Furthermore, very little is known about the mechanism of imprinting-associated methylation and therefore it is possible that patterns could be replicated, at least in part, by continuous *de novo* methylation. The expression profile of *DRM2::GUS* during seed development highlighted the potential role of this enzyme with respect to imprinting-associated methylation in the endosperm.

6.3.3.6 The *DRM2::GUS* transgene exhibited paternal silencing in seed until approximately 3DAP

Seed that inherited the *DRM2::GUS* transgene from the maternal parent exhibited localized GUS activity within the suspensor 1DAP (due to time limitations seed at earlier stages of development were not tested for GUS activity). In contrast, seed that inherited the *DRM2::GUS* transgene from the paternal parent did not show detectable GUS activity until 3DAP. Again the staining was localized to the suspensor. This data is consistent with the previously discussed theory that most of the paternal genome

may be silenced in early seed development (Vielle-Calzada et al., 2000) and raises the possibility that MET1 could act to repress the expression of other DNA methyltransferases, along with other loci, from the paternal genome.

6.3.3.7 DRM2 may play a role in maintaining imprinting-associated methylation in the suspensor

The localization of *DRM2::GUS* activity to the suspensor from early development to at least 8 DAP suggests that DRM2 has an active role to play in this structure. The suspensor was originally thought to simply act as an anchor for the developing embryo, however accumulating evidence suggests that the suspensor plays an active role in early development by synthesizing growth factors (Ceccarelli et al., 1981; Cionini, 1987; Piaggesi et al., 1989) and providing a channel for nutrients to move from the surrounding cells or medium to the growing embryo (Yeung, 1980, Ciavatta et al., 2001). Hence, based on the parental-conflict theory of genomic imprinting, the suspensor is a potential target for imprinting. For example, genes that promote suspensor growth or the transport of nutrients through this structure to the embryo may be maternally imprinted (silenced), thus limiting the amount of resources going to individual offspring. In this scenario, DRM2 may act to maintain this imprinted state specifically within the suspensor. However, it should be pointed out that to date no imprinted genes that are expressed within the suspensor have been identified and no parent-of-origin effect on suspensor development was noted in interploidy crosses (Scott et al.,1998). On the other hand, only a few imprinted plant genes are known and many more may remain to be identified. It is also possible that any imbalance of imprinted gene expression within the suspensor may not have an obvious structural effect, but instead alters the influx of nutrients to the embryo.

6.3.3.8 *DRM2::GUS* is expressed in the developing embryo from approximately 3DAP (globular stage) to seed maturity

Expression of a maternally inherited *DRM2::GUS* transgene was first detectable in the embryo 2 to 3DAP. However, when paternally inherited, staining of the embryo was delayed 3 to 4DAP (depending on the transgenic line). GUS staining was then observed in both types of embryo throughout the remainder of seed development. The

DRM2::GUS transgene appeared to be constitutively expressed since GUS activity was detectable in all the embryonic tissues at a very high level. Indeed, often so much precipitate was produced that the isolated embryos (dissected out of the seed to allow direct contact with the histochemical reagent) annealed to the bottom of the Petri dish. A shorter incubation of the embryos with histochemical reagent would allow more detailed analysis of any specific expression of the transgene in the embryo, which may have been masked by the intense staining. This high level of expression in the embryo suggests that DRM2 could have an important function during embryonic development. If, as predicted, *DRM2* encodes a *de novo* DNA methyltransferase, this may suggest that new methylation patterns are continually added to the embryo during development.

It should also be noted that the tissues around the suspensor and the embryo, including the micropylar endosperm, also frequently stained for GUS activity. This may suggest that DRM2 is involved in the maintenance of imprinting-associated methylation in this section of the endosperm. However, on occasion the maternally derived integuments also exhibited positive GUS staining, even when the *DRM2::GUS* transgene was inherited from the paternal parent. This indicates that the staining of these tissues was due to the leaching of stain from the embryo and suspensor. Thus it is unlikely that DRM2 has a role in propagating imprinting-associated methylation in the endosperm during seed development.

6.3.3.9 A role for DRM2 in the establishment and propagation of imprinting-associated methylation

Whether DRM2 participates in the parent-of-origin effect in *A.thaliana* remains to be determined. However the expression studies described in this Chapter imply that DRM2 is at least expressed in many of the tissues in which imprints are likely to be established or maintained. Of particular interest is the localization of *DRM2::GUS* to the suspensor highlighting the fact that this structure has been neglected in the study of plant imprinting. For example, the analysis of suspensor-specific genes could help to identify more imprinted genes in *A.thaliana.*

6.3.4 *MET1* and *DRM2* exhibit contrasting patterns of expression during floral and seed development

The contrasting patterns of expression of the *MET1::GUS* and the *DRM2::GUS* transgenes indicate a requirement for different spatially diverse DNA methyltransferases during plant development. For example, the *MET1::GUS* transgene showed early activity in the embryo, while the *DRM2::GUS* transgene was active in the suspensor of seed at a similar stage of development. A complementary pattern of expression was also observed in the vascular tissues of the flowers. In *MET1::GUS* flowers showed staining in the anther filament vascular tissue, whilst *DRM2::GUS* flowers exhibited GUS activity in the sepal and petal vascular tissue. Such defined and mutually exclusive patterns suggest that the establishment and propagation of DNA methylation during plant development may be even more complex than previously thought. Only the study of all the putative DNA methyltransferases in an organism will allow us to be able to unravel the function and mechanism of this important epigenetic modification.

6.3.5 Future Work

The roles of MET1 and DRM2 in the parent-of-origin effects in *A.thaliana*, and in development as a whole, remain largely unresolved. However, a more detailed study of the *MET1::GUS* and *DRM2::GUS* lines will help to define these roles. The analysis of more transgenic lines would allow the clarification and verification of the expression profiles of both of these enzymes. Particular emphasis should lay in the development of techniques to permit the study of transgene expression during different periods of gamete development. Furthermore, the possibility that MET1 is subject to paternal imprinting in the CE provides sufficient interest to develop a method of sectioning seeds to observed the maternal expression of the *MET1::GUS* transgene in the endosperm and embryo. The exciting observation that DRM2 is expressed in the suspensor of developing seed has highlighted the possibility that this structure could be subjected to genomic imprinting. Future studies should include the parent-of-origin expression of genes expressed within the suspensor, in a view to identifying imprinted genes.

Chapter 7

A screen to identify mutants in sex-specific components on the genomic imprinting system

7.1 Introduction

7.1.1 A search for the missing pieces of genomic imprinting

Genomic imprinting in plants continues to be an intriguing puzzle, with a multitude of the pieces still missing. Although the evidence described in this thesis supports the model that DNA methylation plays a global role in imprinting, how alleles are sex-specifically marked remains a mystery. Furthermore, only a handful of imprinted genes have been characterised in plants (Kermicle and Alleman, 1990; Lund et al., 1995a,b; Kinoshita et al., 1999; Vielle-Calzada et al., 1999) and at the date of writing no genes have been identified as maternally imprinted (paternally expressed). Isolating more genes that are imprinted, or involved in the imprinting mechanism, would provide us with a greater insight into this fascinating epigenetic phenomenon. In a practical sense it could provide us with excellent tools with which to manipulate imprinting, for the reasons outlined in length in Section 1.5.

7.1.2 The concept of a screen to isolate imprinted genes and genes involved in the imprinting mechanism

To identify such genes we designed a screen based on the parent-of-origin effect on seed size observed in interploidy crosses (Haig and Westoby, 1991;Scott et al., 1998). As described in detail in Section 1.3.3, in crosses between 2x and 4x plants, when the 4x plant is used as the maternal parent, [4x X 2x], seed are produced with a mass significantly smaller than the wild type 2x equivalent. In this case extra maternal genomes are contributed to the endosperm. In contrast, seed from the reciprocal cross [2x X 4x], have a larger mass than the wild type 2x control. Here extra paternal genomes are contributed to the endosperm. It is predicted that this alteration of seed

mass is a direct result of an imbalance in the expression of imprinted genes, affecting endosperm development (Scott et al., 1998).

The reciprocal seed phenotypes observed are in accordance with the Haig and Westoby Parental Conflict Theory of imprinting (Haig and Westoby, 1989, 1991; Moore and Haig, 1991). As discussed in greater depth in Section 1.3.4 this predicts that the female genome silences (imprints) genes that promote endosperm development. In contrast the paternal genome silences (imprints) genes that limit endosperm development.

As described in detail in Chapter 3, the parent-of-origin effect on seed mass can be phenocopied, without the addition of extra parental genes, if one of the parents has a demethylated genome. A [*MET1a/s* X 2x] cross gives seed larger than a wild-type 2x control, whilst [2x X *MET1a*/s] seed are significantly smaller. The model outlined in Figure 3.16 states that demethylation relaxes imprinting and this leads to the expression of otherwise silenced (imprinted) genes. The result after fertilization is again an imbalance in imprinted gene expression, which is reflected in the mature seed mass. Hence, even without the physical addition of extra parental genomes, an imbalance in imprinted genes can be achieved.

In theory, if a factor involved in sex specific imprinting is misexpressed, this could result in a change in seed mass. If a plant carries a null mutation in a gene required for the silencing of maternally imprinted loci, this could result in an increase in seed mass due to the over expression of normally silenced endosperm promoting genes from the maternal genome (Figure 7.1 B). In this case the altered seed mass phenotype would be expressed only if the mutation was inherited through the maternal parent, as the factor is specific to the maternal imprinting system.

Similar observations have already been made for the FIS complex genes (*MEA*, *FIS2* and *FIE*). The products of these genes have been predicted to control the silencing of imprinted loci in the maternal genome (Vinkenoog et al., 2001) Mutations in these genes not only confer a degree of autonomous endosperm development (without fertilization) but the mutant phenotype is only inherited through the maternal parent (Ohad et al.,1996; Chaudhury et al.,1997; Ohad et al.,1999; Kiyosue et al.,1999; Luo

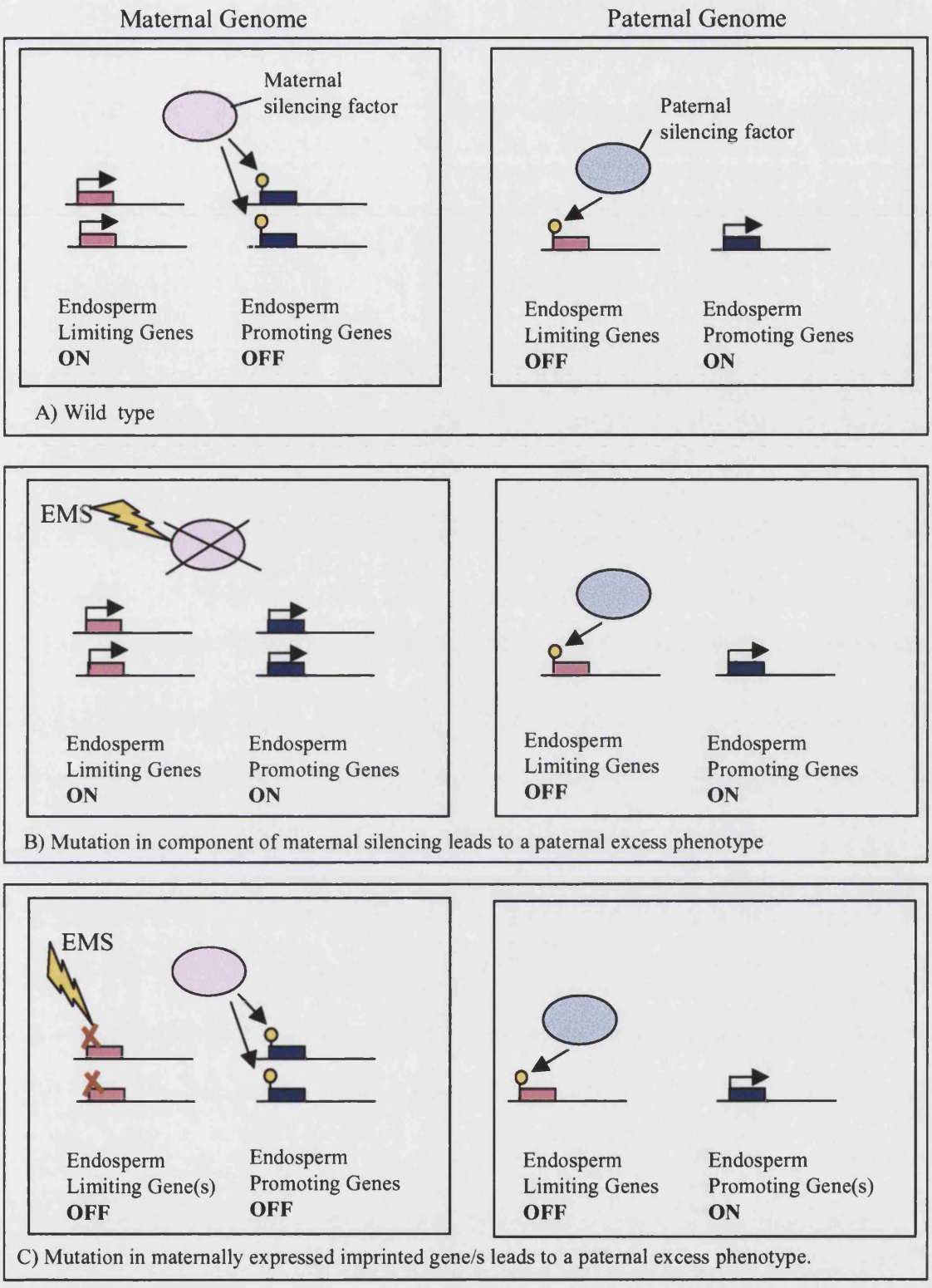


Figure 7.1

Model of mutations in the sex specific genomic imprinting system that could result in a **paternal excess** seed phenotype. (A) A model of the components of a wild type imprinting system. (B) A mutation in a component of the maternal silencing system results in the expression of normally silent endosperm-promoting genes from the maternal genome. This results in a paternal excess imbalance in gene expression and a paternal excess (large) seed phenotype. (C) A mutation in a maternally expressed endosperm-limiting gene reduces or abolishes expression. This leads to a paternal excess imbalance of imprinted gene expression and results in a paternal excess (large) seed phenotype.

et al.,1999). Furthermore, pollination of either a *mea-3* (putative loss of function allele), *fis 2* or *fie* ovule with wild type pollen results in seed abortion (Peacock et al., 1995; Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus et al.,1998). The peripheral endosperm of these seed is highly proliferated and uncellularized. This may be interpreted as an extreme imbalance of imprinted gene expression (similar to [2x x 6x] cross) due to the release of silencing of endosperm promoting genes (Scott et al., 1998). Thus a less drastic effect on a factor of maternal silencing could lead to viable over proliferation of the endosperm and a subsequent increase in seed mass. In contrast, a null mutation in a gene essential for the silencing of endosperm limiting genes, this time in the paternal genome, could lead to seed with a significantly reduced mass (Figure 7.2 B). As the factor is specific for paternal silencing, the phenotype would only be expressed when the mutation is inherited though the pollen parent.

These are only two scenarios for an alteration in seed size. The loss of monoallelic expression of single imprinted genes (either by the loss of repression or loss of function) could have a direct effect on mature seed size. The example shown in Figure 7.1 C shows the loss of expression of an endosperm-limiting gene from the maternal genome. This results in a paternal excess imbalance of imprinted gene expression and a larger seed size. Again, as this is a factor of the maternal imprinting system the altered seed phenotype will only be expressed if the mutation is inherited through the maternal parent. The reverse situation, the loss of expression of an endosperm-promoting gene from the paternal genome, is shown in Figure 7.2 C. Here the imbalance in imprinted gene expression leads to maternal excess and a small seed size. Alternatively, mutations in genes not involved in genomic imprinting could result in an alteration in mature seed mass. For example, mutations in genes involved in nutrient transfer, metabolic pathways or the cell cycle, to name but a few, could result in smaller or larger seed, depending on the role of the encoded gene product.

Hence a number of tests are required to determine the nature of any new mutations. Firstly, the alteration of seed size needs to be expressed in the next generation (i.e. a heritable change). Secondly, if the mutation affects a factor of imprinting the seed phenotype should be expressed in a parent-of-origin specific manner when the candidate plants are used in reciprocal crosses with wild type plants. Furthermore, the

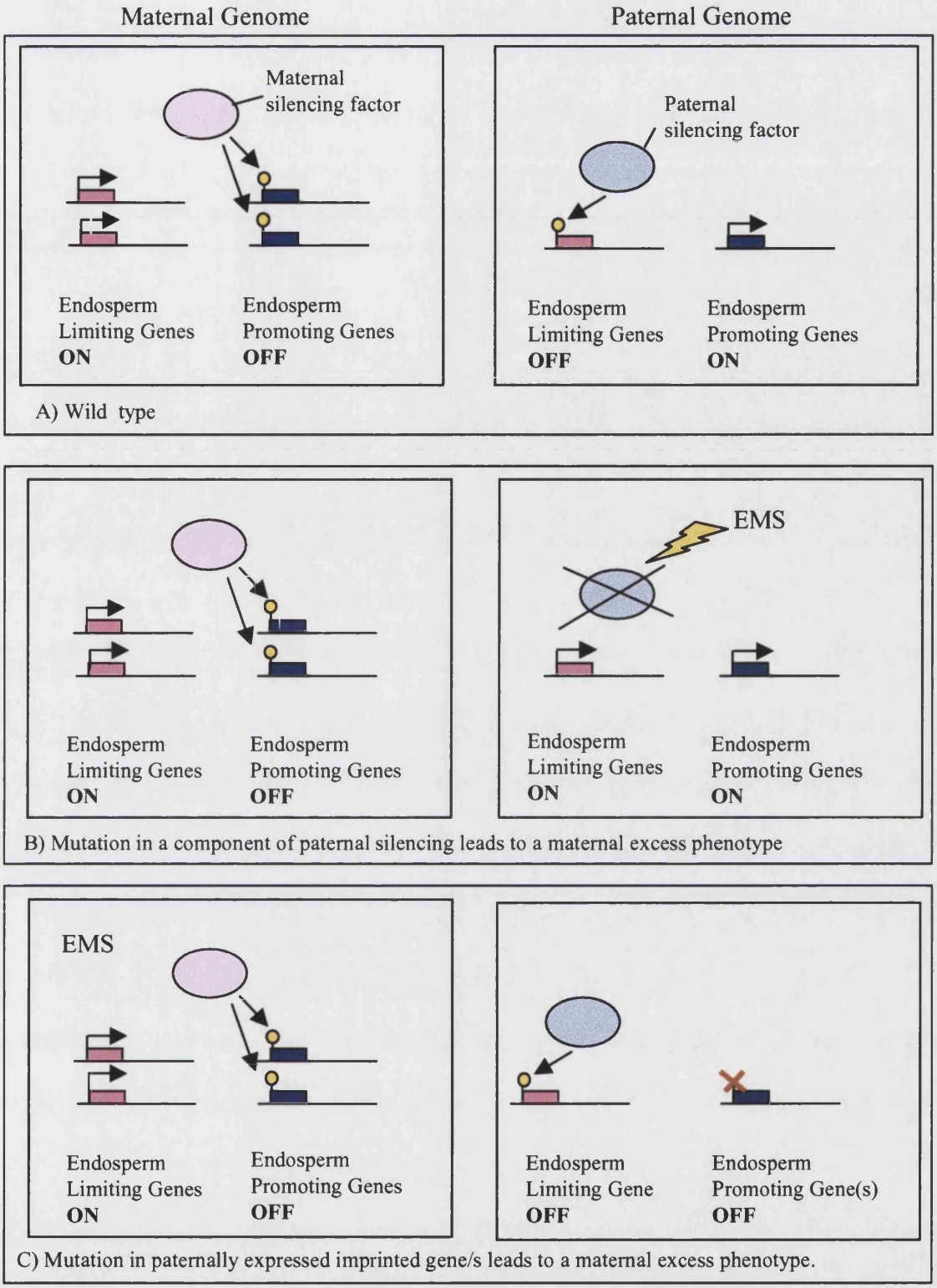


Figure 7.2

Model of mutations in the sex specific genomic imprinting system that could result in a **maternal excess** seed phenotype. (A) The components of a wild type imprinting system. (B) A mutation in a component of the paternal silencing system results in the expression of normally silent endosperm limiting genes from the paternal genome. This results in a maternal excess imbalance in gene expression and a maternal excess (small) seed phenotype. (C) A mutation in a paternally expressed endosperm - promoting gene reduces or abolishes expression. This leads to a maternal excess imbalance of imprinted gene expression and results in a maternal excess (small) seed phenotype.

model predicts that if the altered seed phenotype is the result of an imbalance of imprinted gene expression in the endosperm, a corresponding endosperm phenotype should be observed. That is seed with a mass that phenocopies a maternal excess cross (small) should have reduced endosperm proliferation. On the other hand, seed isolated with a paternal excess-like (large) seed phenotype should show an over proliferation of the endosperm.

Therefore the objectives of the work described in this Chapter were as follows.

1) To design a screen that allowed the efficient separation of candidate seed from a mutagenized population that had mature masses either smaller (maternal excess phenocopy) or larger (paternal excess phenocopy) than a wild type control.
2) To analyse if the altered seed phenotype was expressed in the next generation.
3) To test if the altered seed phenotype was expressed in a parent-of-origin specific manner in crosses with wild type plants.
4) To analyse if the altered seed phenotype correlated with an increase in endosperm proliferation.
5) To test the nature of the inheritance of the mutation.

7.2 Results

7.2.1 The design of a screen for mutants in the sex-specific imprinting system

The objective of the work was to isolate mutants in the imprinting system, based on the hypothesis that a change in sex-specific imprinting will lead to a uniparentally expressed alteration in mature seed mass. The first aim was to identify seed that had a mass larger (phenocopy of paternal excess) or smaller (phenocopy of maternal excess) than a wild-type control. The design of this screen was based on the following principals.

1) The seed population chosen for the screen should have been treated with a suitable mutagen.
2) The mutagenised seed population chosen should contain a larger percentage of relatively small or large seed, in comparison to a control, than a wild-type population.
3) The method chosen to screen the mutagenised seed should effectively separate seed with different mature masses.

7.2.1.1 EMS treated seed contained a higher percentage of small or large seed than a wild type control population

Seed treated with the mutagen, ethylmethane sulfonate (EMS), was chosen for the screen. High quality seeds pre-treated with EMS can be obtained commercially (LEHLE SEEDS) so there was no risk of exposure to this harmful chemical. We proposed to screen the seed for an alteration in seed mass using a series of sieves of different mesh diameter. To be successful this required that as mesh size increased so did the average mass of the seed retained. The sieve mesh diameters chosen for the screen were 393, 355, 335, 250, 200 and 150μm, as the sieve size 355μm was already successfully used in other experiments to separate seeds from other plant debris. It was predicted that the small changes in sieve size would be sufficient to separate seed with different masses. To test this a population of seed was sieved, first with the largest mesh size (393μm). All seed retained in this sieve were put aside and the remaining seed sieved with the next mesh size in the series (355μm). This was

repeated until all the sieves had been used. A sample of seed (3 sets of 10 seeds) was weighed for each group and the average mass of the seed retained calculated for that mesh size (Figure 7.3). An increase in the mesh diameter of the sieve correlated with an increase in average mass of the retained seed, confirming that the sieving method was an efficient way to separate seeds of different masses.

The next requirement of the method was that the mutagenised EMS seed population should contain a higher percentage of small or large seed than a wild-type control population. This would indicate that mutagenesis could produce mutants potentially affected in imprinting, although we did anticipate a high level of noise due to mutations which affected seed size but were unrelated to genomic imprinting. A small sample of 100 mutagenised seed was sieved as described above and the number of seed retained was recorded. This process was then repeated for a sample of 100 wild-type seed (Figure 7.4).

The range of sieves was successful in discriminating between wild type and mutagenised seed. The 355, 335, 300, 250 and 200μm sieve sizes retained seed from the mutagenised population, whilst the wild-type population had seeds retained only in the 300 and 250μm mesh sizes. No seed was retained for either population, in 393 and 150μm mesh sizes. The EMS treated sample population therefore contained seeds that were larger and seeds that were smaller in mass, than the wild type sample population. Extrapolated to the rest of the EMS mutagenised seed population this suggested that a large number of candidate seed, with an altered seed mass relative to a wild-type control, could be identified by this method. The method therefore had good prospects for isolating mutants in the sex-specific imprinting system.

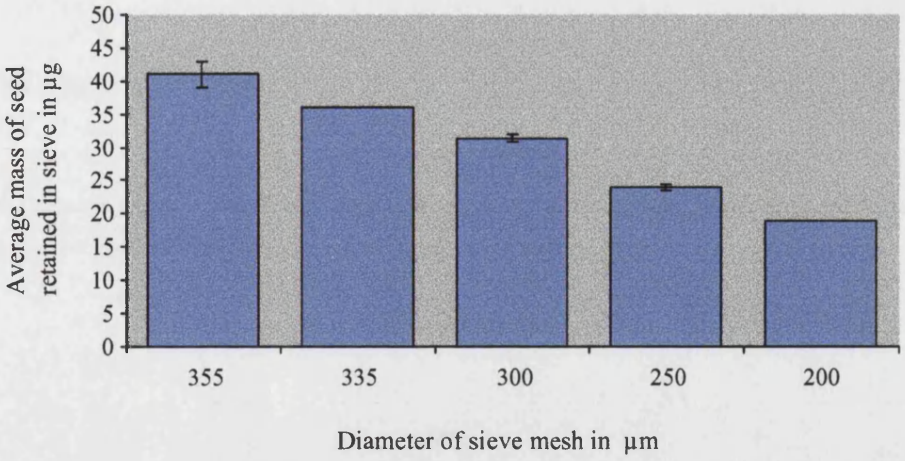


Figure 7.3

The average mass of seed retained in each sieve size from a wild type population. The 393µm sieve was not included as only one seed was retained in this sieve and our method could not accurately weigh a single seed. The standard errors are shown as vertical bars.

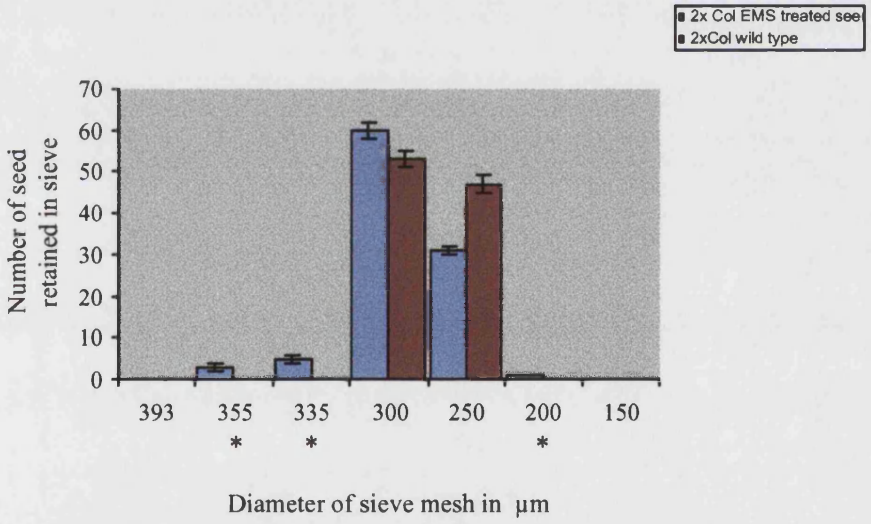


Figure 7.4

The number of seed retained in each sieve size for a sample of 100 EMS treated seed and 100 2xCol seed. No seed were retained in the 393 or the 150µm sieves. The sieves which retained seed only from the EMS population are highlighted with a star.

7.2.1.2 The identification of sieve diameters that would isolate paternal excess and maternal excess candidates from the EMS treated seed population

The next stage of method development required the selection of sieve sizes that would allow the identification of paternal excess candidates (large) and maternal excess candidates (small). Seed from a maternal ([4xC24 X 2xC24], 21μg) and a paternal excess cross ([2xC24 X 4x C24], 42μg) that had been generated in previous experiments were used to calibrate the sieve sizes. To perform the calibration these seed, tetraploid seed (4x Col, 35μg) and diploid seed (2x Col, 22 μg) were sieved in the manner described above. The C24 ecotype was used for the parental excess crosses as no seed from Col interploidy crosses were available at the time.

Seed from both the paternal excess cross [2xC24 X 4xC24] and wild type 4xCol seed were retained in the 393, 355 and 335μm mesh sizes (Figure 7.5). As no wild-type 2xCol seed were caught in these sieves the 393, 355 and 335μm sieves were used to separate paternal excess (large) candidates from an EMS population. All the seed tested were retained in the 250μm mesh diameter and therefore the 200 and 150μm in sieves were used to isolate maternal excess (small) candidate seed. Hence, the sieving method allowed a large number of EMS treated seed to be efficiently screened for alterations in seed size.

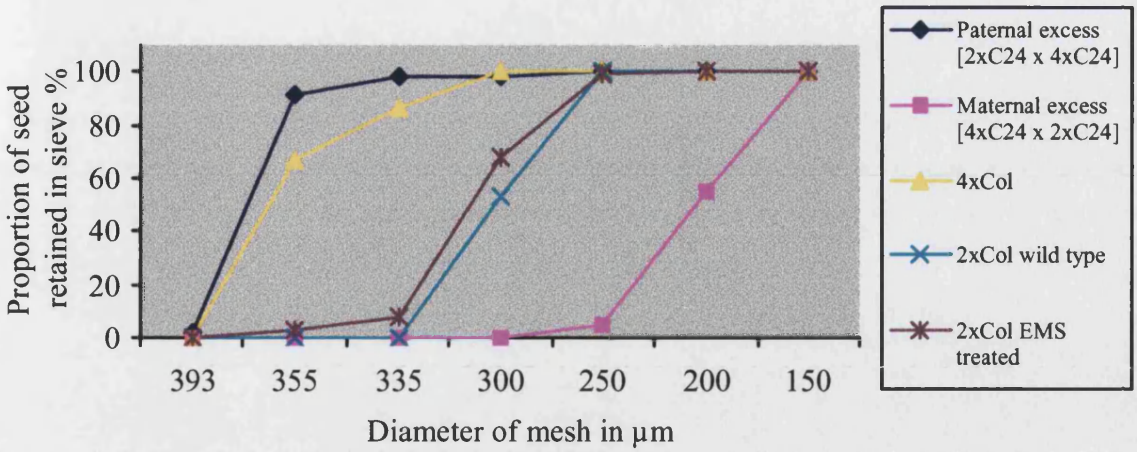


Figure 7.5

Calibration of the sieves to identify which mesh size will isolate maternal excess (small) and paternal excess (large) candidates from an EMS treated 2x Col population.

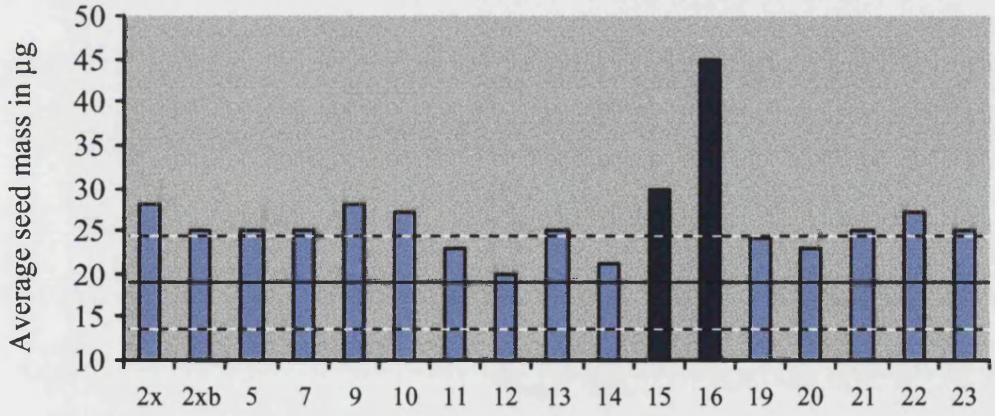


Figure 7.6

The mature mass of seed from candidate plants in parental group (PG) 22 isolated in the screen for paternal excess mutants. The average mass of 2xCol seed (from all trays) is shown as the solid horizontal line and the ±s.d as the dotted lines. 2x and 2xb represent the average mature mass of seed from the 2 control 2xCol plants in the PG 22 tray itself. Plants 15 and 16 (in dark blue) were chosen as candidate plants as they maintained a large seed mass in comparison to the 2xCol controls.

7.2.2 The screen for mutants in the sex-specific imprinting system

7.2.2.1 The isolation of paternal excess and maternal excess candidates from an EMS treated seed population by sieving

Approximately 77 000 seed were processed in the first round of screening (Table 7.1). Seed retained in the sieve sizes, 393, 355 and 335μm were identified as paternal excess candidate mutants, whilst seed retained in the sieve sizes 200 and 150μm were identified as maternal excess candidate mutants. As each parental group may have contained seeds from the same mutation event each group was screened separately. This allowed seed to be tracked throughout the screen and could give early clues if two candidate lines were the progeny of the same mutation event.

Table 7.1

An example of the number of seed caught in each sieve diameter for 2 parental groups

Sieve mesh diameter in μm	Parental group 3 seed distribution	Parental group 7 seed distribution
393[a]	1	2
355[a]	13	54
335[a]	100 [c]	150[c]
300	3250[c]	3600[c]
250	1375[c]	1050[c]
200[b]	50	175
150[b]	8	14

[a] Sieve to separate candidate paternal excess-like mutants
[b] Sieve to separate candidate maternal excess-like mutants
[c] Estimated number of seed

A large number of candidates were isolated from each parental group (Table 7.1). For the paternal excess (large) candidate screen over 300 seed, collected from the 3 sieve sizes, were identified as potential candidates for each parental group. Seeds isolated in the 393μm sieve often appeared damaged, with either broken seed coats or protruding embryos. Most seed caught in the 355μm mesh was intact, and all were used in the next round of screening. Approximately 100 to 200 seed were isolated in the 335μm sieve from each parental group. Due to time and space limitations only a sample of 20 seed, of this latter set were taken into the second round of screening.

Barnaby Speller, a final year undergraduate student under my daily supervision, was mainly responsible for the screen for maternal excess (small) candidates. Approximately 50 to 250 candidate seeds were isolated for each parental group (data not shown). All the seed retained in the smaller 150μm sieve, and a sample of 20 seed from the larger 200μm sieve, were used in the second round of screening.

7.2.2.2Confirming that candidate plants expressed the altered seed size phenotype in the next generation

The aim of the second step of the screen was to test if candidates isolated in the first screen maintained the altered seed mass in the next generation. Furthermore, it ensured that the alteration in seed size was reproducible under the growth conditions described in Section 2.2.1. Candidate seeds from the initial sieving screen were isolated, sown and allowed to self-pollinate to give M3 seed. If the M3 seed maintained the alteration in seed mass compared to a wild type control the candidate lines were characterised further.

7.2.2.2.1 The screen for paternal excess candidates

Mature seed was collected from each candidate plant. Seed were also collected from the 2xCol and 4xCol control plants in each tray and their average mass calculated (see Table 7.2).

Table 7.2

The average mature mass of seed from control 2xCol and 4xCol plants

Ploidy of control plant	Average seed mass in μg ± s.d.	Number of plants tested
2x	22 ± 3.80	11
4x	38 ± 2.40	6

A sample of 10 seed from each candidate plant was weighed and the average mature seed mass obtained. Mature seed mass was then plotted for each plant in each parental group. The average mass for the control Col 2x seed was also plotted horizontally on the same graph, complete with horizontal lines to represent the standard deviation. This allowed the variability between trays to be taken into consideration. With the aid of this graph, candidate lines were chosen that maintained the altered seed size. These were considered the confirmed candidate lines.

A typical graph is illustrated in Figure 7.6 and shows the average mature seed mass for lines isolated in the first round of screening from parental group 22. 13 candidate plants had a seed mass larger than the average 2xCol control of 22 μg. The control 2xCol plants for that particular tray also had a larger mass than the average 2xCol control. Most striking is plant 16, which had seed with a mass of 45μg, over double that of the average 2xCol control. The line 22.16 (parental group 22, candidate M2 plant 16) was therefore chosen as a confirmed candidate line. In total, 141 paternal excess candidate plants were tested in the second step of screening (Table 7.3). Of these 18 (12.8%) were confirmed as maintaining a mature seed mass larger than the 2x Col control. The seed mass of these confirmed paternal excess candidate lines ranged from 28 μg to 45 μg.

Table 7.3

The number of candidate plant lines from each parental group that expressed the altered seed mass in the next generation

Parental Group	Number of plants in second screen[1]	Number of candidate plants[2]	Candidate plant reference[3]	Mass of mature seed in μg[4]
1	8	0		
3	14	2	3.13	31
3			3.15	32
5	6	1	5.2	36
7	16	2	7.9	36
7			7.10	33
8	4	2	8.1	35
8			8.3	37
9	2	0		
11	11	0		
18	13	2	18.7	30
18			18.10	39
22	15	2	22.15	30
22			22.16	45
30	9	2	30.2	32
30			30.6	31
37	15	2	37.5	31
37			37.14	33
38	10	1	38.10	37
39	14	2	39.9	32
39			39.11	28

[1]The number of plant lines that were identified in each parental group as paternal excess candidates (large seed mass) by the sieving method and taken through to the second round of screening.
[2]The number of plant lines for each parental group that were confirmed as maintaining the expression of the altered seed phenotype in the next generation.
[3]The reference number of each confirmed paternal excess candidate line.
[4]The average mature mass of seed from each confirmed paternal excess candidate line.

7.2.2.2.2 The screen for maternal excess candidates

Seed from the screen for maternal excess candidates (small) exhibited extremely poor viability, with a germination rate of approximately 10%. Candidate plants were tested for the stable inheritance of the altered seed phenotype in the next generation in the same way as described for the paternal excess candidates. No lines were identified that produced seed with a mass smaller than the wild type control (data not shown).

7.2.2.3 Determining if the altered seed phenotype is expressed in a parent-of-origin dependent manner

The model on which the screen is based states that mutations in sex-specific imprinting will not only result in a change in seed size, but that the altered seed size phenotype will be expressed in a parent-of-origin specific manner. Therefore the candidate lines were crossed with wild-type plants, in both directions, to examine if any lines showed this parent-of-origin specific dependent expression of the seed phenotype.

7.2.2.3.1 Reciprocal crosses between confirmed candidate lines and 2xCol plants

Approximately 3 to 5 plants of each confirmed candidate line were used in the following experiments. Multiple plants were required as the nature of each mutation was unknown. For example, the M3 plants confirmed as candidates in the second round of screening might have been either homozygous or heterozygous for the mutation in question. The plants tested in this step of the experiment were grown from the self-pollinated seed of these M3 plants. If the M3 plants were homozygous for the mutation all the M4 plants should also be homozygous for the mutation. However, if the M3 plants were heterozygous for the mutation 25% of the M4 plants will not carry the mutation. If too small a sample of plants were tested the mutation might be lost, along with the associated phenotype.

In the preliminary experiments the 'wild type' plants used in the crosses were of the ecotype C24, as a transgenic line was available carrying a barnase transgene (Paul et al., 1992). This transgene confers male sterility, which is advantageous as mechanical emasculation is time consuming and incomplete removal of the stamens can lead to a small degree of self-pollination. Candidate plants were crossed with these 2x C24 plants in reciprocal crosses and mature seed collected and weighed.

Table 7.4

The average mass of seed from reciprocal crosses between paternal excess candidate lines 18.10 and 22.16 and wild type 2xC24 plants

Cross	Average mass of seed in μg
2xC24 x 2xC24	36
18.10 x **18.10** [a]	31
18.10 x 2xC24[a]	30
2xC24 x **18.10**[a]	41
22.16 x **22.16** [b]	42
22.16 x 2xC24 [b]	38
2xC24 x **22.16** [b]	44

[a] Crosses with mutant line 18.10 (parental group 18, plant 10)
[b] Crosses with mutant line 22.16 (parental group 22, plant 16)

A parent-of-origin effect on seed size was observed in crosses with some lines (for example line 18.10 in Table 7.4). In crosses with 2xC24 plants, if line 18.10 was used as the paternal parent the seed were 37 % heavier than when the line was used as the maternal parent. In contrast, seed from both crosses between 2xC24 and line 22.16 gave seed with a mass similar to that of the self-pollinated [22.16 X 22.16] seed. Neither of these observations was in accordance with the model (Figure 7.1) that an altered (large) seed phenotype, caused by a mutation in a maternal specific component of the imprinting machinery, would only be expressed if the mutation were inherited through the maternal parent.

A clue to the unexpected results may lie in a possible maternal effect, via the C24 parent (Alonso-Blanco and Koornneef, 2000), masking the parent-of-origin dependent expression of the altered seed phenotype. It was therefore decided to repeat the experiments with a wild type Col parent. A single line, 22.16 (parental group 22, plant 16), was chosen to repeat the experiments. This was to ensure that crosses with Col wild type parents would indeed give clearer results, as these crosses were more time consuming, due to the need for mechanical emasculation of the Col plants (no male sterile plants were available). The 22.16 line was chosen as it gave a seed with a mass of approximately 42μg, almost double the mass of the 2xCol seed. It was hoped that as the mass of 22.16 seed is so much larger than the wild type this would allow any parent-of-origin dependent expression of the altered seed phenotype to be tracked

with greater ease than a more subtle change in mass (for example line 18.10). Furthermore, as the seed mass of 22.16 is so altered in comparison to the wild type it was predicted that this line could carry a mutation in a locus important for genomic imprinting.

7.2.2.3.2 The parent-of-origin dependent expression of the altered seed phenotype of candidate line 22.16

The candidate line 22.16 was crossed with wild type 2xCol plants in both directions, and the mature seed collected and analysed. When the line 22.16 were used as the maternal parent the seed had a mass of 42μg and exhibited an elongated phenotype compared to the wild type control (Figure 7.7). This was comparable to the phenotypes observed in self-pollinated [22.16 X 22.16] seed. However, if the line 22.16 were used as the paternal parent, [2xCol X 22.16], the seed were similar in shape and mass to the wild type control [2xCol X 2xCol].

Hence the altered (large and elongated) seed phenotype of the line 22.16 was only evident in seed from crosses with 2x Col plants when the line 22.16 was used as the maternal parent. This is in accordance with the model (Figure 7.1) that a seed phenotype resulting from a mutation in a component of the maternal imprinting system would only be expressed were the mutated allele maternally inherited.

7.2.2.4 Interploidy crosses between the candidate line 22.16 and 4xCol plants

The purpose of the next set of crosses was to further examine the parent-of-origin dependent expression of the altered seed phenotype. Were the altered seed phenotype observed in 22.16 seed caused by a paternal excess imbalance in imprinting the model predicts that the addition of extra paternal genomes to the endosperm (for example by a [22.16 X 4xCol] cross) would cause a more extreme imbalance in imprinting, perhaps leading to seed abortion. Previous work on the outcome of interploidy crosses had reported a [2x X 4x] cross as producing viable seed (Scott et al., 1998). The model (Figure 7.1) goes on to predict that the reciprocal cross, [4x Col X 22.16], would produce seed with a phenotype comparable to seed from a [4xCol X 2xCol]

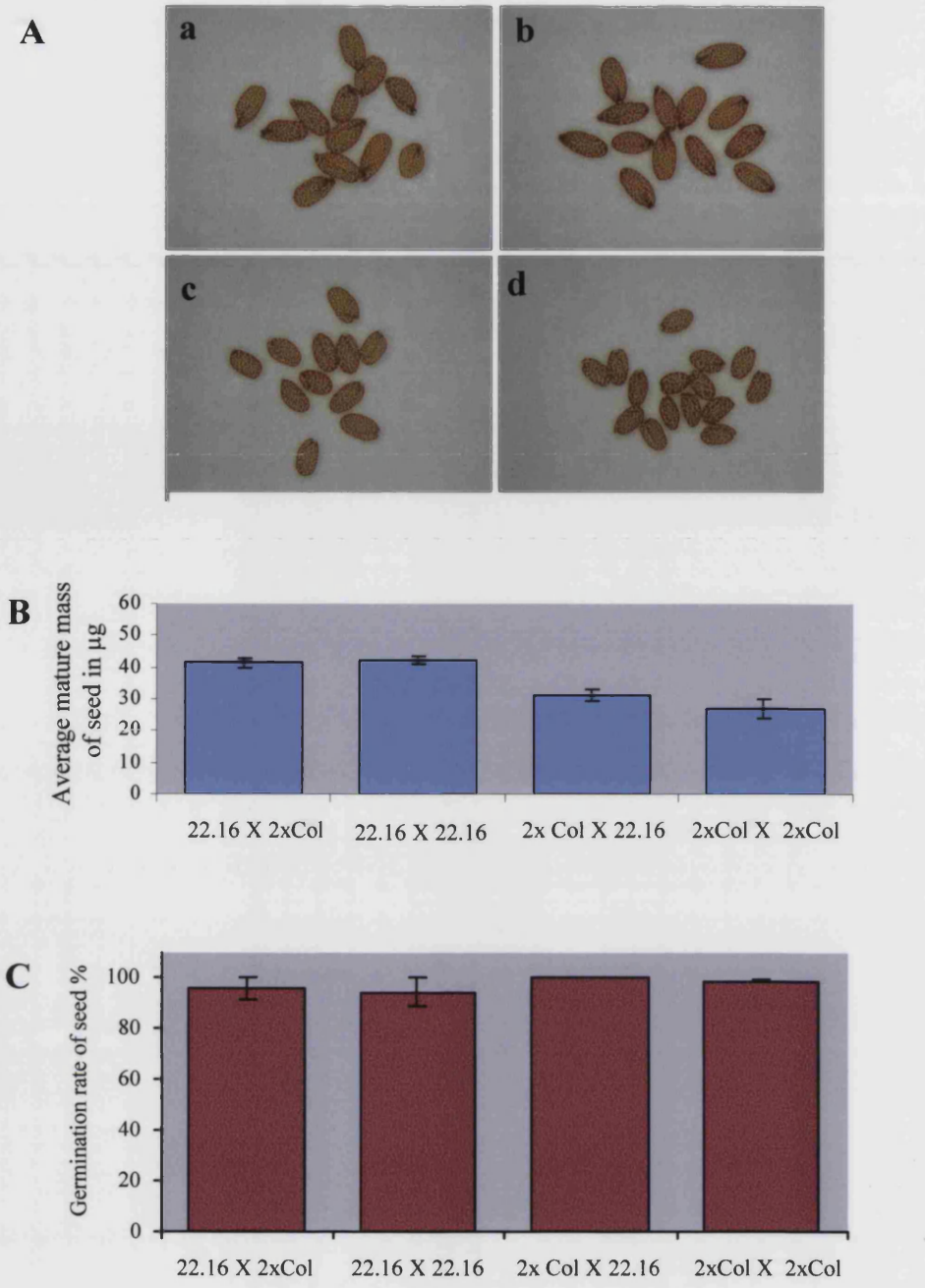


Figure 7.7

The phenotype of mature seed from the reciprocal crosses between the paternal excess candidate line22.16 and 2xCol. (**A**) Seed from a [22.16 x 22.16](a) and a [22.16 x 2xCol](b) cross were larger and more elongated in shape than seed from either a [2xCol x 22.16] (c) or a [2xCol x 2xCol]cross (d). (B) Shows the average mass of seed from the reciprocal crosses. Seed with a maternal 22.16 parent were heavier than seed with a 2xCol maternal parent. The seed from all 4 crosses showed a high rate of germination (**C**). In (**B**) and (**C**) the bars represent the s.d.

cross, as the altered seed phenotype is only expressed if the 22.16 line is used as the maternal parent.

The line 22.16 was crossed with 4xCol in both directions and the mature seed and analysed for mature mass, germination rate and shape phenotype. The maternal excess crosses [Col4x XCol2x] and [Col4x X 22.16] both gave plump, round seed with a high rate of germination (over 84%) (Figure 7.8). In accordance with the predictions of the model (Figure 7.1) both had a similar mature seed mass (19μg and 20μg respectively).

In contrast, seed from the cross [2xCol X 4xCol] had a low germination rate of 29% (Figure 7.8). If this paternal excess cross was repeated with the line 22.16, [22.16 X 4xCol], the seed obtained had a slightly larger mass (26μg compared to 20μg) and a slightly lower viability (16% compared to 29%). The [22.16 X 4xCol] seed also exhibited the elongated seed phenotype.

One clear difference between the experiments described here and the published results of Scott et al., (1998) was the ecotype used in the crosses. The experiments conducted by Scott et al., (1998) had used the ecotypes C24 and Landsberg *erecta* (Ler), both of which produce viable seed from a [2x X 4x] cross. The ecotype used here was Col.

7.2.2.5 The ecotype effect on seed development in interploidy crosses

The unexpected low germination rate of [2xCol X 4xCol] seed led to the design of experiments to examine the differences between ecotypes with respect to the development of seed produced from interploidy crosses. Hence the crosses described in the last section were repeated with the both C24 and Col ecotypes as the wild type 2x and 4x plants. Mature seed were collected and mature mass and rates of germination tested.

The results for the maternal excess crosses are shown in Figure 7.9. For clarity the C24 parent has been highlighted for each cross. The 4 permutations of crosses, [4xCol X 2xCol], [4xCol x **2xC24**], [**4xC24** x 2xCol] and [**4xC24 x 2x C24**] gave

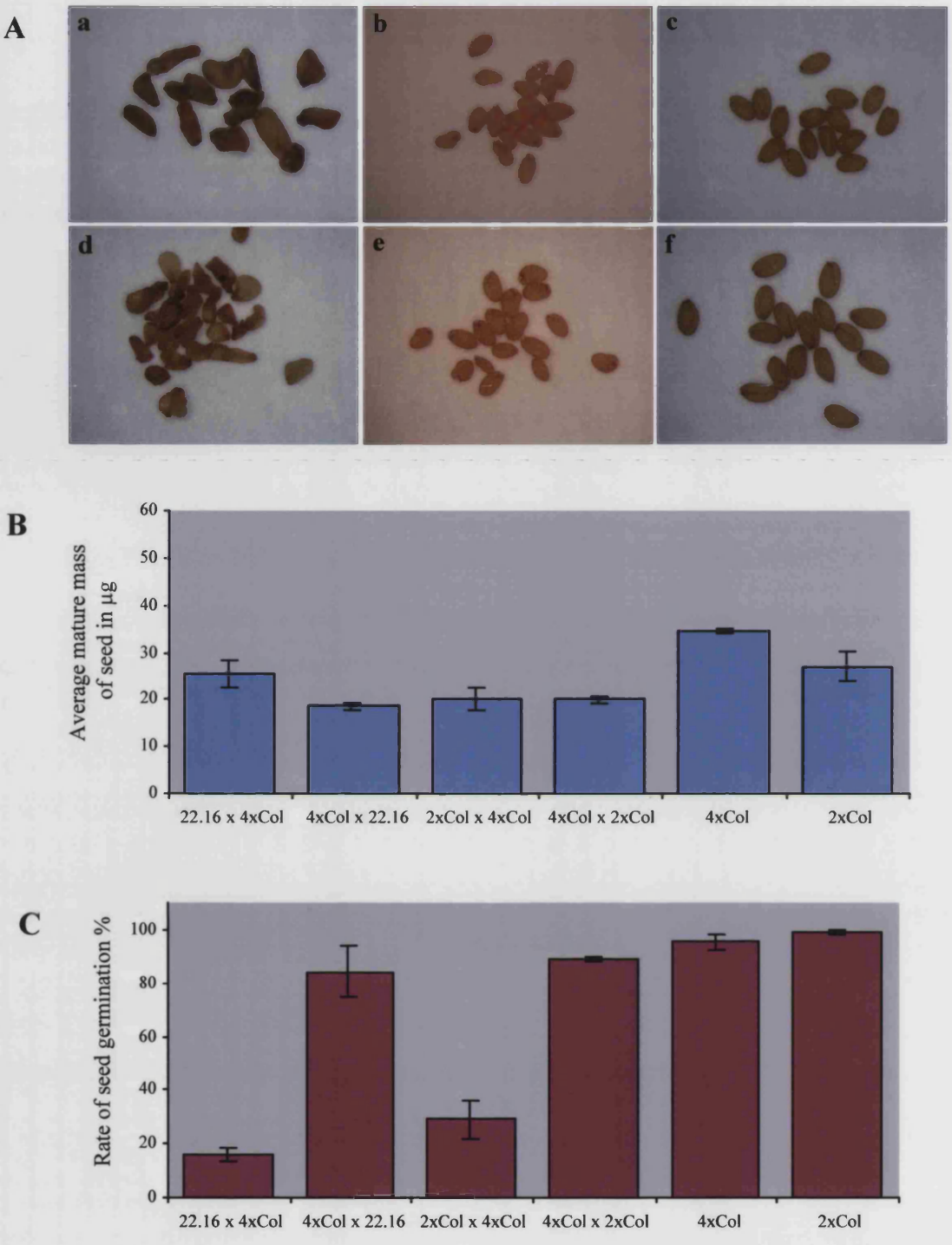


Figure 7.8

The phenotype of seed from interploidy crosses between 22.16 and 4xCol. (**A**) (a)[22.16 X 4xCol], (b) [4xCol X 22.16], (c) 2xCol, (d) [2xCol X 4xCol], (e)[4xCol X 2xCol] and (f) 4xCol. (**B**) shows the average mature mass of seed and (**C**) shows the rate of germination for each cross. The bars represent the s.d.

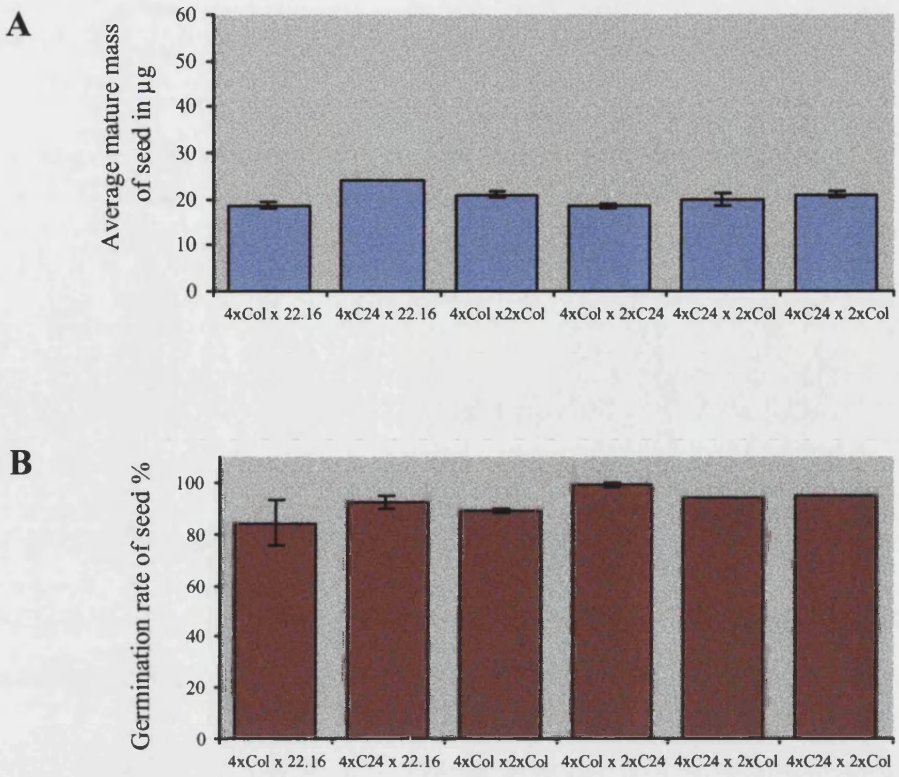


Figure 7.9

The mature mass (**A**) and viability (**B**) of seed from maternal excess interploidy crosses between the candidate line 22.16 and 4xCol and 4xC24 plants.

seed with similar mature mass (19-21μg) and rate of germination (89-95%). Thus there was little difference if either 4xCol or 4xC24 was used as the maternal parent.

In contrast, differences were observed in the equivalent paternal excess crosses. As outlined above, seed from a [2xCol X 4xCol] cross had a significantly lower mass (20μg) and viability (29%) than those resulting from a **[2xC24 X 4xC24]** cross (seed mass 49μg, germination rate 100%). Strikingly, when the 4x paternal parent was replaced with the C24 ecotype, [2xCol X **4xC24**], the seed had germination rate and mass similar to a **[2xC24** X **4xC24]** (Figure 7.10). When the cross was repeated with a replacement of the 2x maternal parent **[2xC24** x 4xCol], although on average seed had mass of 46μg, viability was only 50%. In summary, replacing either the maternal or paternal parent in a [2xCol X 4xCol] cross with the equivalent C24 parent resulted in a significant increase in seed size and viability. However, the increase in viability was more pronounced if the replacement was the tetraploid paternal parent.

7.2.2.6 The ecotype effect on developing seed from interploidy crosses with the paternal excess candidate mutant line 22.16

The experiments outlined in section 7.2.2.5 were extended in order to clarify the results obtained with the crosses between 22.16 and 4x plants. The [22.16 X 4xCol] cross produced a low percentage of viable seed (16%). In isolation this observation supports the proposal that the mutation carried by 22.16 affects the maternal imprinting system. However, the high frequency of seed abortion from a [2x Col X 4xCol] cross suggested that the low germination rate of seed from a [22.16 X 4xCol] cross could be due to the 4xCol parent as opposed to the maternal 22.16 parent. Hence the line 22.16 was also crossed with 4xC24 and 4xCol plants, in both directions, and the resulting seed analysed for their mature mass and rate of germination. If 4xCol was used as the maternal parent, [4xCol X 22.16], the seed had a mass of 19μg and an average germination rate of 84% (Figure 7.9). However, if the C24 ecotype was used, **[4xC24** X 22.16], the seed were slightly heavier (24μg) and showed a small increase in viability (95%).

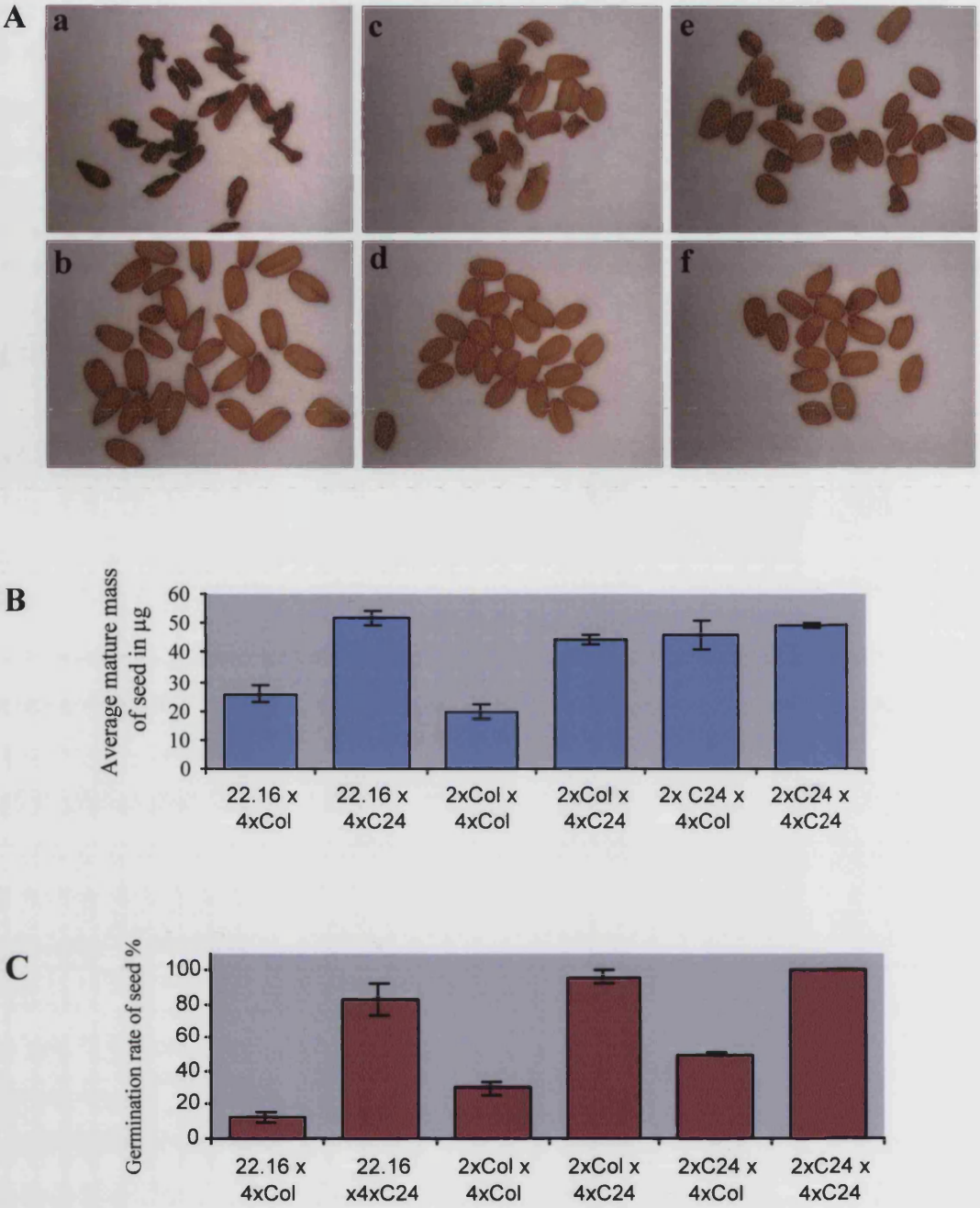


Figure 7.10

Seed from paternal excess interploidy crosses between 22.16 and both 4xCol and 4xC24 plants. (A) The morphology of seed from (a) [22.16 X 4xCol], (b) [22.16 X 4xC24], (c) [2xCol X 4xCol], (d) [2xCol X 4xC24], (e) [2xC24 X 4xCol] and (f) [2xC24 X 4xC24] crosses. The average mature mass(**B**) and viability (**C**) of the seed from each cross. In (**B**) and (**C**) the s.d. is represented by the bars.

The differences were more significant in the paternal excess crosses (Figure 7.10). Here, were the C24 ecotype used as the 4x paternal parent, instead of the Col ecotype, the resulting seed had both a dramatic increase in viability (83% compared to 13%) and a larger mass (52μg compared to 26μg). The seed from the [22.16 X **4xC24**] cross also showed the long elongated seed phenotype.

7.2.3 Further characterisation of the paternal excess candidate line 22.16

7.2.3.1 The 22.16 mutant plants are diploid (2n=10)

The line 22.16 had been identified in the screen for mutants in the imprinting system, via its paternal excess-like seed phenotype. In simple terms, due to the fact 22.16 seed had a larger mass than wild type Col2x seed. To eliminate the possibility that the altered seed phenotype was due to a change in chromosome number, chromosome counts were conducted on squashed inflorescence meristem tissue of 22.16 plants. All the 20 cells counted (from 3 separate plants) contained 10 chromosomes.

7.2.3.2 The development of the embryo and endosperm in seed from the paternal excess candidate 22.16 in comparison to wild type

The aim of the next set of experiments was to analyse endosperm development in the paternal excess candidate 22.16.A prediction of the model on which the screen was based (Figure 7.1) is that paternal excess imbalance promotes endosperm development with seed from a paternal excess cross (for example [2xC24 x 4xC24]) exhibiting an over proliferation of the endosperm. This over proliferation includes a greater number of PE nuclei, a delayed cellularization of the PE, a larger CE cyst and more chalazal nodule. Hence, if the 22.16 line carries a mutation in the maternal genomic imprinting system the seed should thereby show an over proliferation of the endosperm. Furthermore, if the mutation is in a maternal specific factor of imprinting then the altered endosperm phenotype should only be expressed in seed inheriting the mutant 22.16 locus from the maternal parent.

7.2.3.2 .1 Endosperm development in 22.16 seed

The development of the endosperm was studied in seeds from 22.16 and 2xCol plants at different stages of seed development using confocal microscopy. Figure 7.11 shows seed at the heart stage of development. All 22.16 seed at this stage exhibited a thin elongated CE in comparison to the characteristic dome shape of the CE cyst in the 2xCol control. In total, 3 classes of CE abnormality were observed in the 22.16 seed. The first class, (Figure 7.11, seed C) had a CE that, although elongated, had a similar volume to that of the 2xCol control. The second class (Figure 7.11 seed B) contained unusual fibrous structures, in this case physically linking the CE to a large chalazal nodule. The final class of 22.16 seed (Figure 7.11 seed A) had a CE that was severely misshapen, with diffuse cytoplasm. The time point of PE cellularization (initiated at the early heart stage of development) was comparable in 22.16 and 2xCol seed.

In later stages of development it was difficult to observe either the embryo or the CE in 22.16 seed, as the seed integuments were often apparent in the same plane as these structures, obscuring the image. However, Figure 7.12 shows later CE development from the few seeds where images could be captured. Although, clear pictures of the embryos could not be taken an estimation of embryo stage was possible.

The CE of the 22.16 seed was larger than that seen in the earlier stage seeds and retained the constricted phenotype. In the majority of cases the CE had limited contact with the maternal chalazal proliferating tissue (CPT) (Figure 7.12 A and B) and the embryo in these seed had reached the late heart stage of development (data not shown). Interestingly, although cellularizied PE was noted in 22.16 seed at heart stage (Figure 7.11) it was difficult to observe PE development in these seed after 6 DAP. However, abnormal round structures were present throughout the central cell of these later stage 22.16 seed, which were not observed in the wild type control (Figure 7.13). These structures were larger in later stage seed (compare Figure 7.13 B and C) and only showed evidence of cellularization in less than 10% of the seed (Figure 7.13 A).

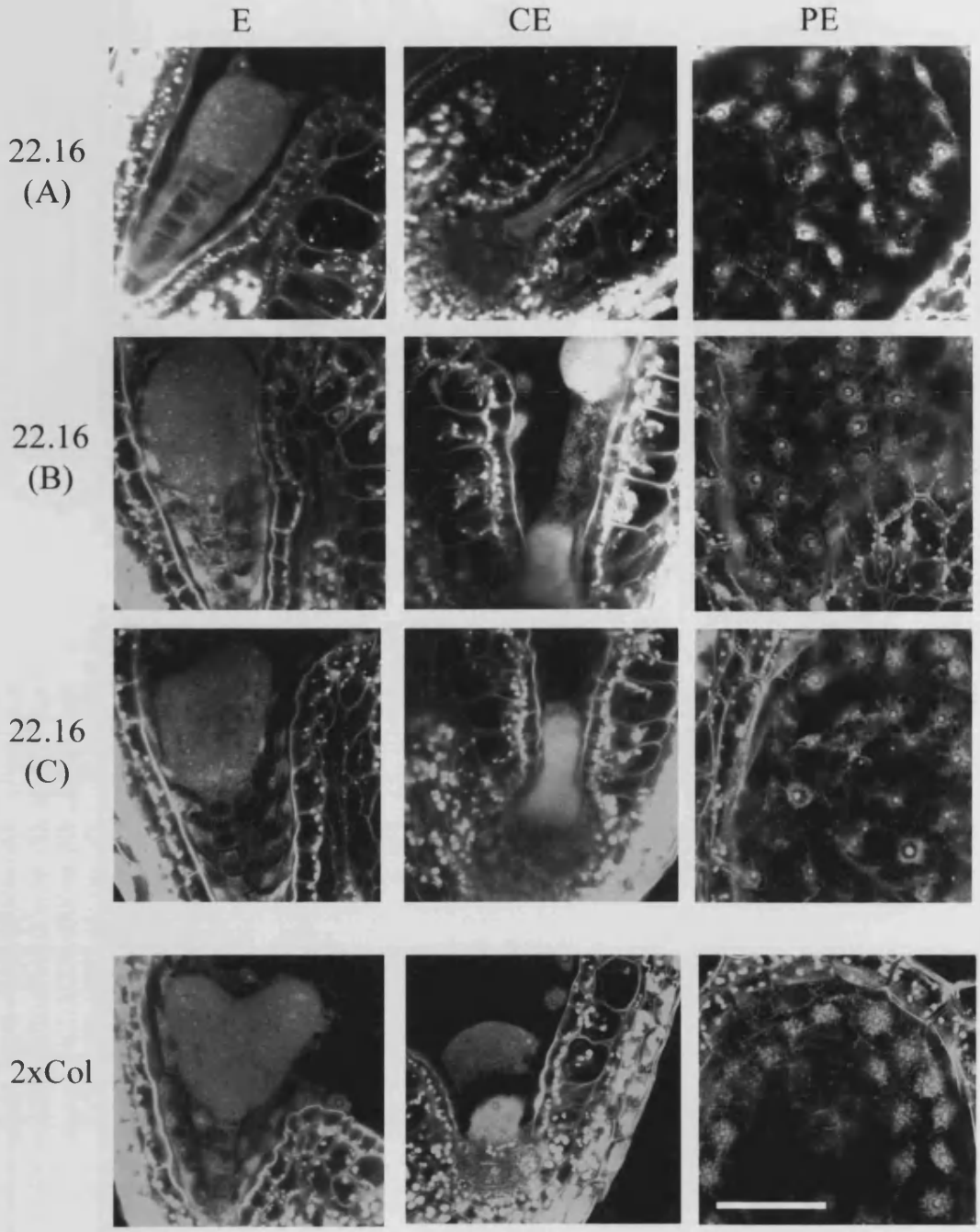


Figure 7.11

Endosperm development in 22.16 and 2xCol seed at the heart stage of embryo development. 3 types of CE were observed in 22.16 seed (A, B and C). All 3 types had an elongated morphology compared to the wild type 2xCol equivalent. The PE of 22.16 seed was similar in morphology and time point of cellularization to that in 2xCol seed. E, embryo; CE, chalazal endosperm; PE, peripheral endosperm. Bar, 50um.

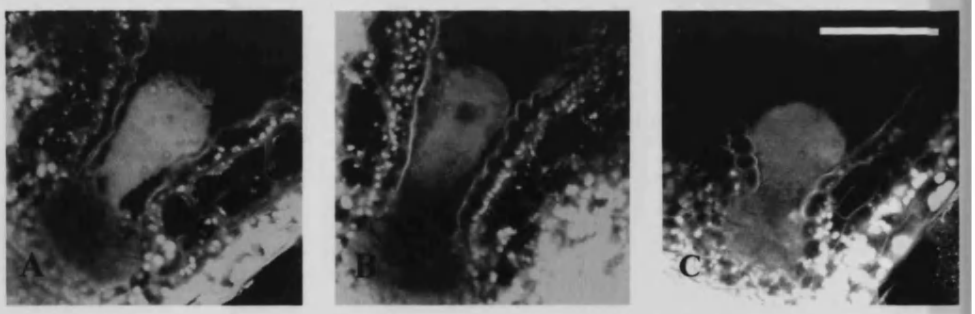


Figure 7.12

CE in 22.16 seed (A and B) and 2xCol seed (C) 6 DAP. The CE of 22.16 seed was elongated in comparison to the wild type. At the basal foot of the CE in 22.16 seed, there was little contact with the CPT (A) or the cytoplasm was diffuse (B), Bar, 50um.

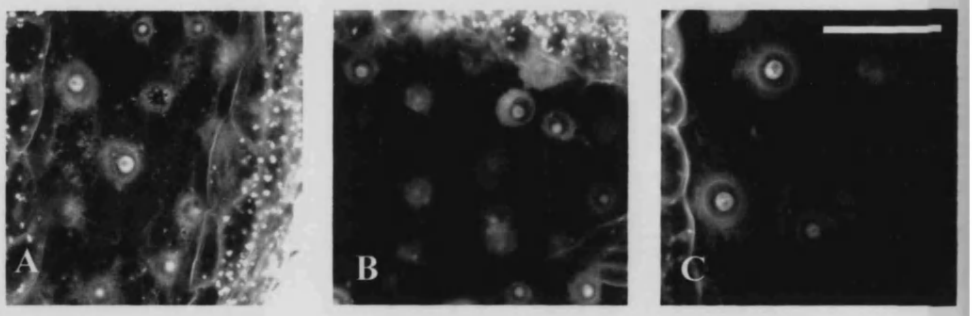


Figure 7.13

The large nucleate structures observed in the central cell of 22.16 seed 6DAP (A and B) or 7DAP (C). A proportion of seed showed signs of cellularization (A) although the majority (B and C) did not. Bar, 50 um.

7.2.3.2.2 Endosperm development in seed from reciprocal crosses between 22.16 and 2xCol plants

Were the endosperm phenotype observed in 22.16 seed due to a disruption of a maternal specific component of the imprinting system, (causing a paternal excess imbalance of imprinting in the endosperm), the phenotype should be expressed only if the mutation is maternally inherited. This theory was tested by examining the development of the endosperm in seed from reciprocal crosses between wild type and 22.16 plants.

In contrast to seed from a [22.16 X 22.16] cross, progeny from a [22.16 X 2xCol] cross could be observed fully until 7 to 8 DAP. This allowed comparisons in CE and PE development to be made with the reciprocal cross, [2xCol X 22.16] and a wild type control [2xCol X 2xCol], at later seed stages (Figure 7.14). At the heart stage, endosperm development of [2xCol X 22.16] seed was comparable to that of the wild type. The CE was domed with dense cytoplasm and a similar volume to the wild type control. In contrast, seed from the reciprocal cross, [22.16 X 2xCol], had a CE with a larger cross sectional area (and probably a larger volume) than the wild type. The CE also had diffuse cytoplasm and large vacuoles. Thus the altered CE phenotype is only expressed if maternally inherited. However, PE development in seed from the two reciprocal crosses between 22.16 and 2x Col was similar to that of the wild type, at the equivalent stage of seed development.

7.2.3.2.3 Endosperm development in seed from interploidy crosses between 22.16 and 4xCol plants

The following work was to further test if the altered seed phenotype of 22.16 was due to a paternal excess imbalance in imprinting, resulting in an increased proliferation of the endosperm and a larger seed. If additional paternal genomes were added to the endosperm via a [22.16 X 4xCol] cross the model (Figure 7.1) predicts that this would result in further proliferation of the endosperm in comparison to the control [2xCol X 4xCol] cross. The model also predicts that the reciprocal cross, [4xCol X 22.16] would have an endosperm phenotype comparable to that of the control cross

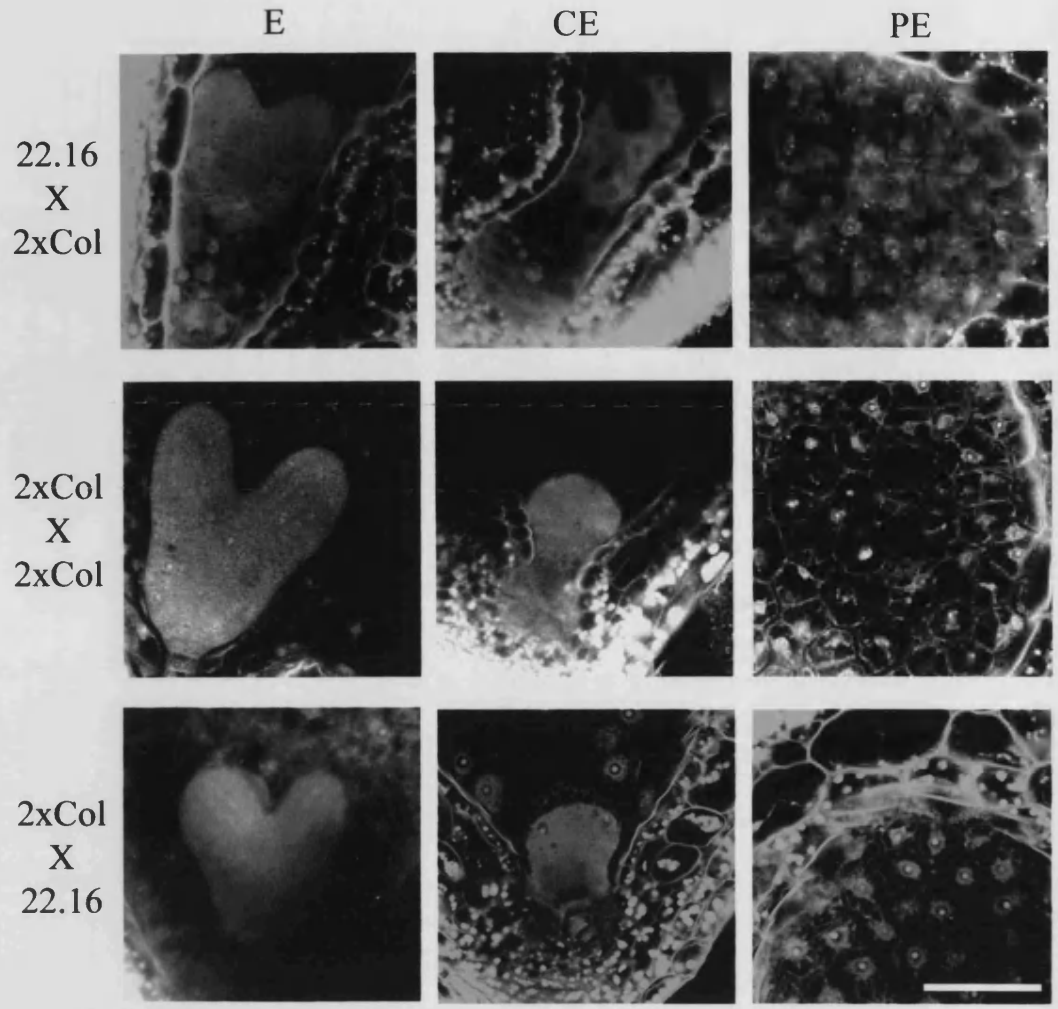


Figure 7.14
Endosperm development in seed from reciprocal crosses between 22.16 and 2xCol plants at the heart stage of embryo development.

[4xCol X 2xCol], as the altered seed phenotype is only expressed if the 22.16 mutant locus is inherited from the maternal parent.

7.2.3.2.3.1 Endosperm development in maternal excess crosses between 22.16 and 4xCol plants

Figure 7.15 shows seed at the globular stage of development from the maternal excess crosses [4xCol X 22.16] and [4xCol X 2xCol]. The endosperm development of these seed was similar. Both had limited CE development, in comparison to the wild type controls, with no production of chalazal nodules. The PE of both maternal excess crosses had cellularizied early, at the globular stage of embryo development, producing large vaculote PE cells. The control 2x and 4x seed showed no cellularization of the PE, at this stage of development.

7.2.3.2.3.2 Endosperm development in paternal excess crosses between 22.16 and 4xCol plants

Seed from the paternal excess crosses [22.16 X 4xCol] and [2xCol X 4xCol] are shown in Figures 7.16 and 7.17. At the globular stage of development (Figure 7.16) the endosperm of both [22.16 X 4xCol] and [2xCol X 4xCol] seed exhibited abnormalities that were similar to those previously observed in the lethal [2xC24 X 6xC24] cross (Scott et al., 1998). The PE nuclei of the seed appeared to be linked in strings, distributed randomly throughout the central cell. The cytoplasm surrounding the nuclei was often stretched forming elliptical structures. In contrast, in the wild type controls the PE nuclei were more evenly distributed throughout the seed and were surrounded by the characteristic sphere of cytoplasm.

2 broad classes of CE development were observed in [22.16 X 4xCol] seed. In the first class (Figure 7.16, seed A) the CE was severely elongated and often had little physical contact with the CPT. A large proportion of these seed also had a collapsed integuments, suggesting that the seed was destined to abort. In the second class of seed the CE was similar in morphology to that of the [2xCol X 2xCol] and [4xCol X

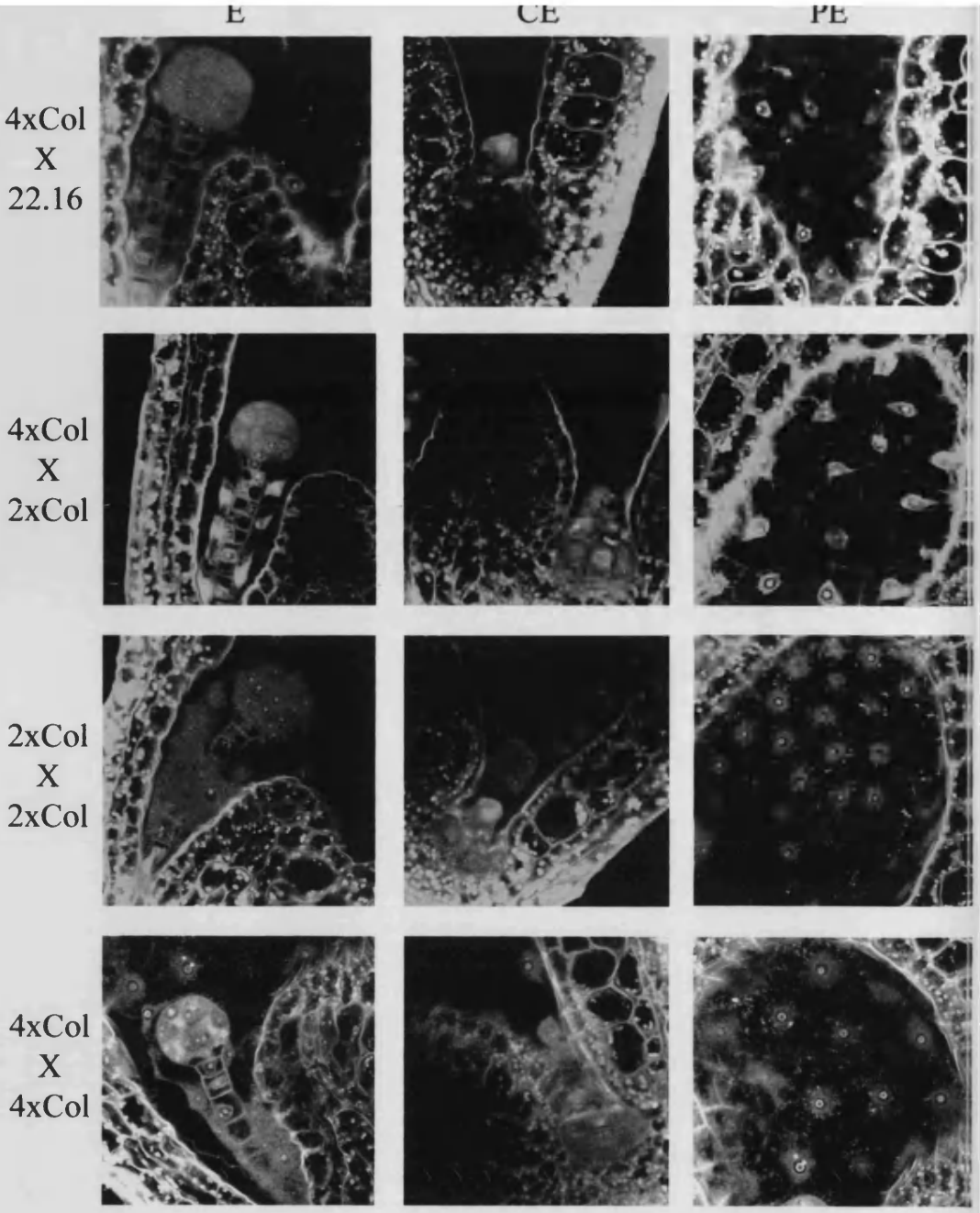


Figure 7.15

Endosperm development in seed from maternal excess crosses with candidate line 22.16 and the ecotype Col. All seed are at the globular stage of embryo development. E, embryo; CE, chalazal endosperm; PE, peripheral endosperm. Bar, 50um.

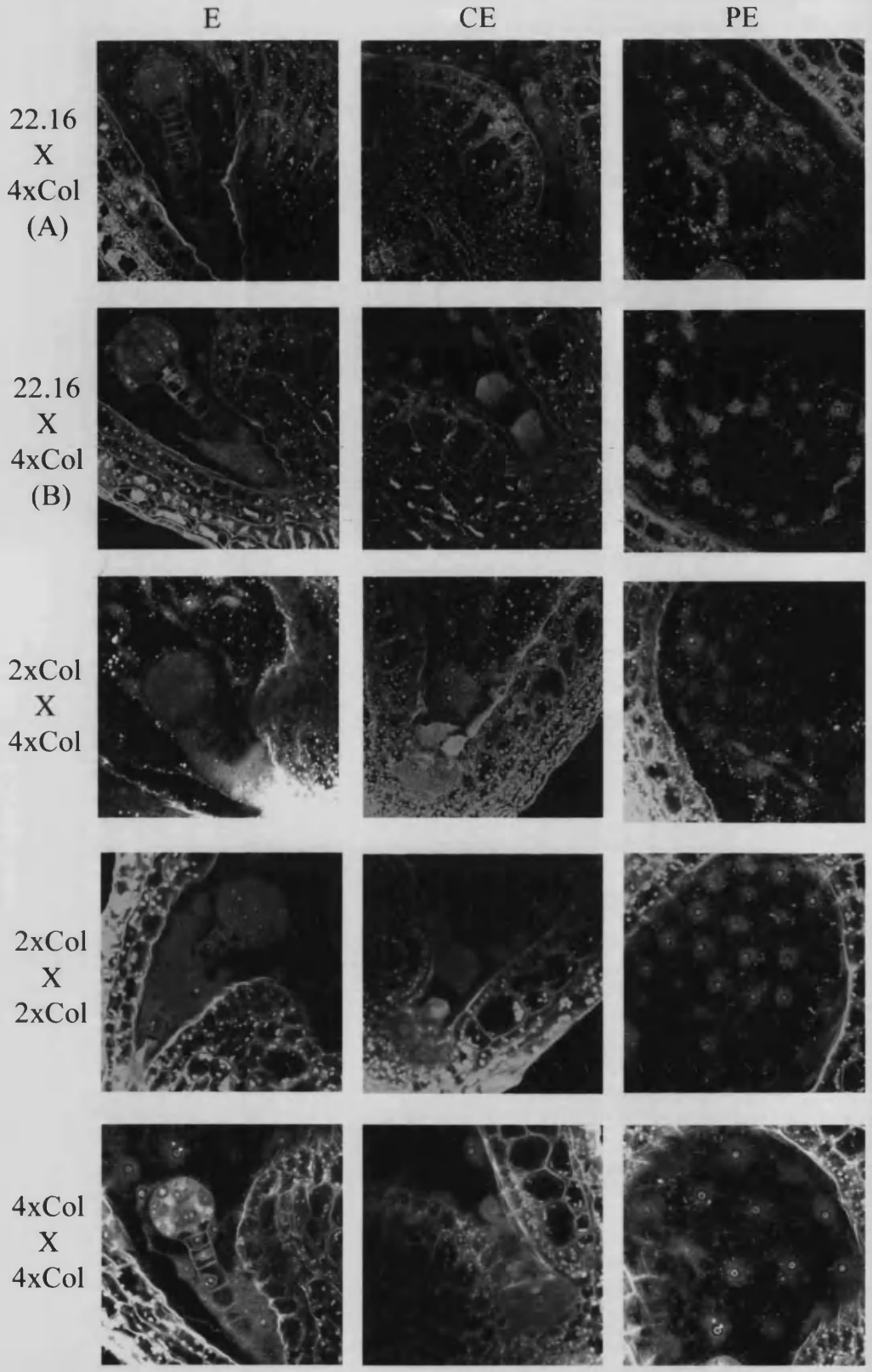


Figure 7.16

Endosperm development in seed from paternal excess crosses between 22.16 and the ecotype Col at the globular stage of embryo development. 2 types of CE morphology were observed in 22.16 seed (A and B). All 22.16 seed showed an abnormal PE, with the nuclei irregularly situated throughout the central cell (compared to 2xCol or 4xCol seed). Seed from the wild type paternal excess cross also exhibited abnormal PE development compared to those from the balanced 2xCol and 4xCol crosses.

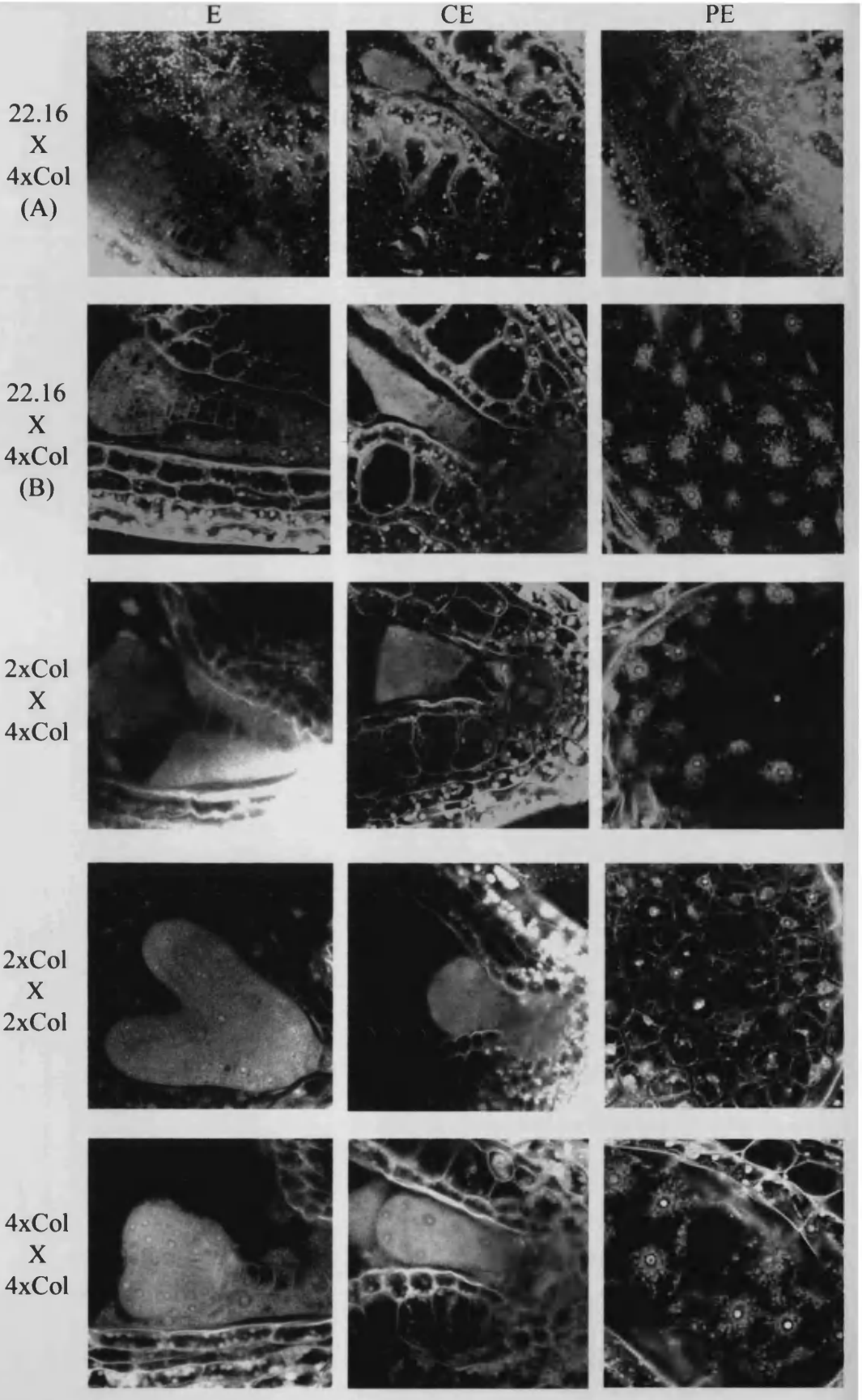


Figure 7.17

Endosperm development in seed from paternal excess crosses between 22.16 and 4xCol plants at the heart stage of embryo development. As in the previous figure the 2 types of endosperm development observed in (22.16 X 4x) seed (A and B) are shown. Bar, 50um

4xCol] controls. The CE in seed from the [2xCol X 4xCol] cross was also similar in size and shape at this stage of development.

Seed at a later stage of development are shown in Figure 7.17. The [22.16 X 4xCol] seed again showed 2 broad classes of CE development. In one class the CE was severely misshapen and detached from the CPT (Figure 7.17, seed A). In the example shown, the CE of the seed had characteristics more reminiscent of an embryo than chalazal tissue. Indeed the elongated region contained structures similar in morphology to suspensor cells. In the second class of seed (Figure 7.17, seed B) the CE, although long and elongated in comparison to the [2xCol X 2xCol] control, still resembled CE tissue. Interestingly, the abnormal endosperm development was also noted in seed from a [2xCol X 4xCol] cross.

The PE of the interploidy crosses, [22.16 X 4xCol] and [2xCol X 4xCol], was not seen to cellularize at any time point tested (Figure 7.17). The strings of PE nuclei, observed at the globular stage of development, were also noted in the later stages of development. Again the severity of endosperm abnormality varied in seed from a [22.16 X 4xCol] cross. In some seed the PE nuclei were severely misshapen and the integuments had collapsed so that they were in the same plane as the embryo (Figure 7.17, seed A). In contrast, the second type of seed (Figure 7.17, seed B) had less abnormal nuclei and the integuments were intact.

Embryo development was delayed in seeds from both a [22.16 X 4xCol] cross and a [2xCol X 4xCol] cross, in comparison to the wild type [2xCol x 2xCol] control. At a similar time after pollination embryos in seed from the paternal excess crosses had only reached the globular stage of development, in comparison to the late heart/early torpedo stage seen in the [2xCol X 2xCol] control. Development of the embryo was also delayed in [4xCol X 4xCol] seeds compared to [2xCol X 2xCol] seed. In seed 6DAP the tetraploid embryo had only reached heart stage in comparison to the late heart/early torpedo stage of the diploid embryo.

7.2.3.3 Comparison of embryo size from mature 22.16 and 2xCol seed

The aim for this step of work was to examine if the altered mass of 22.16 seed was reflected by a change in embryo size. The model predicts that the altered seed phenotype is due to a paternal excess imbalance of imprinting, which results in an increased proliferation of the endosperm. In *A.thaliana* the embryo is thought to derive nutrients from the transient endosperm (Himer et al., 1998) and therefore a larger endosperm should theoretically produce a larger embryo. Embryos were gently extracted from 2xCol and 22.16 fresh mature seed and in accordance with the prediction 22.16 embryos were larger in size than 2xCol control (Figure 7.18).

7.2.3.4 Comparison of ovule and whole seed development in 22.16 and 2xCol plants

The model (Figure 7.1) predicts that the altered seed phenotype of 22.16 seed could be due to a paternal excess imbalance of imprinting. As the primary effect of imprinting is thought to be on endosperm development, the major effect of the mutation should be apparent in the endosperm as opposed to other structures, such as the integuments/seed coat. Hence the aim of this section was to examine at what stage the altered size phenotype could be observed and if the altered size correlated with an increase in endosperm proliferation. As the maternal genome is encased in tissues that will go on to contribute to the mature seed the effect of the mutation might be observed prior to fertilization. Therefore the objectives of this experiment were as follows.

1) To study the development of 22.16 ovules compared to wild type development in 2xCol ovules. The main aim being to study if any alteration in size correlated with autonomous endosperm development.
2) To study early seed development in whole seeds from selfed 22.16 and 2xCol crosses. As the model (Figure 7.1) predicts that the altered endosperm and size phenotypes should only be expressed if the mutant 22.16 allele is inherited through the maternal parent seed from the reciprocal crosses, [22.16 X 2xCol] and [2xCol X 22.16] , were also studied.

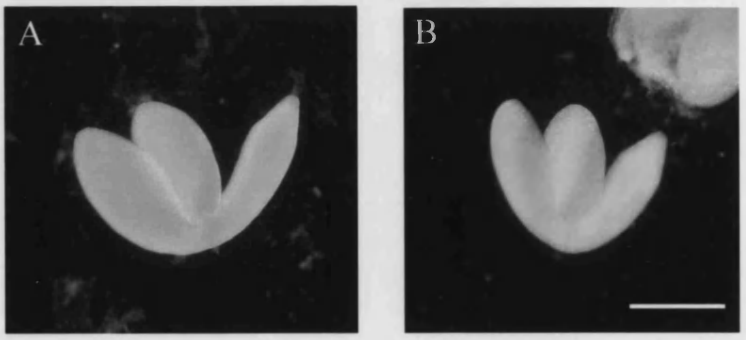


Figure 7.18

Embryos from mature 22.16 (A) and 2xCol (B) seed. Bar, 0.2mm.

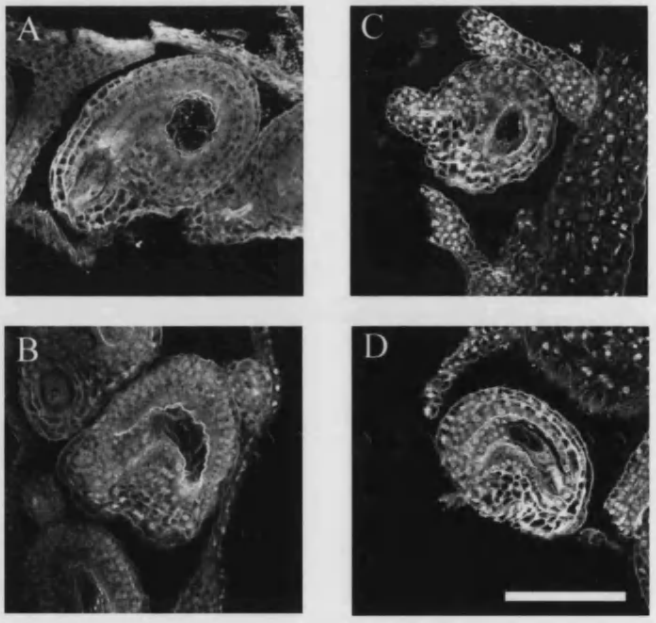


Figure 7.19

Ovules from 22.16 (A and B) and 2xCol (C and D) plants. Bar, 85 um.

7.2.3.4 .1 A comparison of ovule development in 22.16 and 2xCol plants

Ovules from a 22.16 and a 2xCol plant are shown in different orientations in Figure 7.19. The 22.16 ovules exhibited an increase in size in comparison to the 2xCol ovules. No autonomous endosperm development was seen in either the 22.16 ovules or the wild type control.

7.2.3.4.2 A study of whole seed development in 22.16 seed

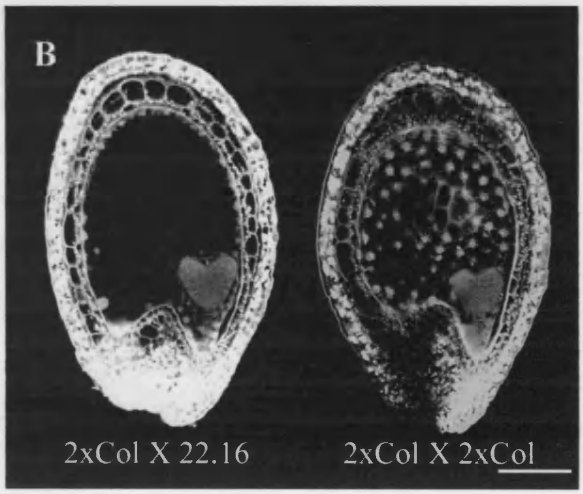
Table 7.5

Measurements of 22.16, 2xCol, [22.16 X 2xCol] and [2xCol X 22.16] seed size at globular and heart stage of development (see Figure 7.20).

Cross	Length of globular stage seed in mm	Width of globular stage seed in mm	Length of heart stage seed in mm	Width of heart stage seed in mm	Number of cells in micropylar half of the inner integument[1]
22.16 X 2xCol	0.68	0.35	N/A	N/A	28
22.16 X 22.16	0.66	0.35	N/A	N/A	25
2xCol X 22.16	0.45	0.28	0.50	0.32	18
2xCol X 2xCol	0.45	0.26	0.50	0.35	18

[1]The number of cells in the chalazal half of the inner integument were not counted as the layer became constricted towards the CP, and therefore accurate and reproducible counts could not be made.

The larger size of 22.16 seed compared to the wild type control was apparent at the globular stage of embryo development (Figure 7.20 A, Table 7.5). The [22.16 X 22.16] seed were approximately 50% longer, and 25% wider, than the wild type [2xCol X 2xCol] control. Seed from the reciprocal crosses, [22.16 X 2xCol] and [2xCol X 22.16], had dimensions comparable to those of their maternal parents at this stage of development. This increase in seed length correlated with an increased



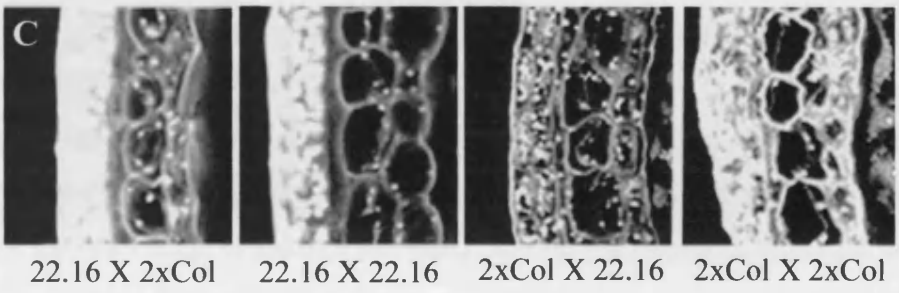


Figure 7.20

Confocal pictures of seed from reciprocal crosses between 22.16 and 2xCol. (A) Seed with a maternal 22.16 parent were larger in size at the globular stage of embryo development than seed with a 2xCol mother. at both the globular stage (A) and the heart stage (B) of embryo development. (C) Further magnification of the integuments using Adobe photoshop showed that the dimensions of these cells was similar in seed with either a 22.16 or 2xCol maternal parent.

number of cells in the (inner) integument (Table 7.5). Both [2xCol X 22.16] and [2xCol X 2xCol] had approximately 18 cells in the micropylar half of the inner integument. In contrast, [22.16 X 22.16] and [22.16 X Col2x] had 28 and 25 cells respectively in the equivalent layer, an increase of 50% compared to the wild type number. The adaxial ridge was also elongated in the [22.16 X 22.16] and [22.16 X Col2x] seed in comparison to the [2xCol X 22.16] and [2xCol X 2xCol] seed (Figure 7.20 A). This resulted in the embryo and chalazal endosperm being compressed within their respective poles. To investigate if the dimensions of cells in the inner integument layer differed between the seeds from the different crosses an equivalent section of the layer was magnified (Figure 7.20 C). Within a single seed the cell dimensions were variable. However, no overall significant differences were observed between cell size in the seed from the different crosses.

The early globular stage of embryo development was chosen as the stage at which to compare 22.16 seed with wild type seed (and their reciprocal crosses) as seed with a 22.16 maternal parent were misshapen in comparison to wild type. This abnormal shape meant the last stage at which a composite picture could be taken, to include both seed poles and no integuments, was the early globular stage. [2xCol X 2xCol] and [2xCol X 22.16] seed reached the globular stage 4DAP, whilst the seed from a [22.16 X 22.16] and [22.16 X 2xCol] cross did not reach the equivalent stage of embryo development until 5 DAP (Figure 7.20 A). It was proposed that 2xCol seed could reach the dimensions of a 22.16 seed after another 24-hour period. In other words, Figure (7.20 A) could represent a delay in 22.16embryo development and not an increase in seed size. Thus composite pictures were taken of seed from both a [2xCol X 2xCol] and [2xCol X 22.16] cross, 5DAP (Figure 7.20 B). Both seed had reached the heart stage of embryo development and had increased in length and width from the globular stage (length 0.50mm, width 0.32mm) (Table 7.5). This was still 0.16 to 0.18 mm shorter in length than seed with a 22.16 maternal parent, 5DAP. Therefore, at the same stage of embryo development seed with a 22.16 maternal parent are still longer than seed with a wild type mother.

7.2.3.5 The floral morphology of 22.16 plants

The 22.16 plants showed a reduced fertility phenotype and we decided to examine this in more detail as it could provide information on the wild type role of the 22.16 product and potentially provide a useful trait for tracking the mutation in mapping experiments. To determine if the reduced fertility was due to defective pollen, ovules or stigma development, the stigmas of 22.16 were pollinated with pollen from the same flowers. In all cases the pod elongated and 95% of ovules were fertilized (data not shown), suggesting that the reduced fertility might be caused by a mechanical defect.

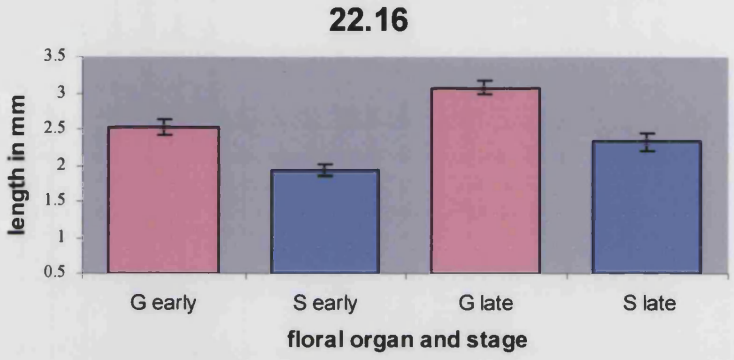
To examine if the reduced fertility was caused by an increase or decrease in length of the stamens or gynociuem the floral organs were measured and compared to wild type. Stamen chosen were one of the 4 medial stamens, as the later initiating lateral stamens can be significantly shorter (Mansfield and Briarty, 1993). Two stages of flower, early and late were used, where early flowers were designated as those that had just opened, whilst late flowers had opened the previous day.

The early stage 2xCol plants had a gynocieum length of 2.0mm and stamens of length 1.7mm (Figure 7.21 B). By the later stage of flower development the stamens had increased in length to be approximately the same size as the gynocieum, allowing pollen to be deposited on the stigma (Figure 7.21 A). In contrast, the early stage 22.16 flowers had a gynocieum 25% longer than the wild type equivalent. The stamens were also slightly longer than the wild type control. Strikingly, the 22.16 gynocieum continued to increase in length up to 3.1mm. The late stage 22.16 stamens also increased in length from the early stage, but were not long enough for pollen to be deposited on the stigma (figure 7.21 A), presumably resulting in the reduced fertility phenotype.

A

22.16 2xCol

B



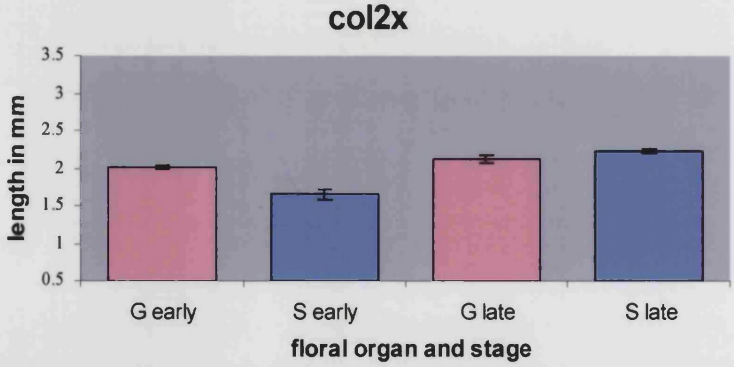


Figure 7.21

Floral morphology of 22.16 plants. 22.16 flowers (A top and bottom left) had reduced fertility with pollen deposited on the ovary walls (see white arrows) as opposed to the stigma as in wild type flowers (A top and bottom right). The floral organs of 22.16 flowers were longer than those in wild type flowers (B). G, gynocieum; S, stamen. in both graphs the bar represents the s.e.m.

7.2.3.6 The genetics of the 22.16 mutation

The candidate line 22.16 was identified in a screen for mutants in the sex specific imprinting system, via its altered (large) seed phenotype. However, mutations in many genes that have no role in imprinting could lead to an altered seed size. Therefore the gene/s or region mutated in the 22.16 candidate line must be identified and characterised before any firm conclusions can be made. Before the mutated region can be mapped a number of questions had to be asked. These were as follows.

1) Was the 22.16 candidate line homozygous for the mutation/s?
2) Was the reduced fertility/floral phenotype inherited as a recessive, dominant or semi dominant trait?
3) Was the altered seed phenotype inherited as a recessive, dominant or semi dominant trait?
4) Were the fertility/floral and seed phenotypes linked, or were they caused by mutations in different genes?
5) Was the altered seed phenotype due to a mutation in a gene with a sporophytic or gametophytic effect?

To tackle the first question, the original 22.16 candidate plant was self-pollinated and seed collected. Approximately 20 plants were grown and tested for the altered seed and floral phenotypes. All the plants showed both the seed and floral traits, suggesting that the 22.16 was homozygous for the mutation/s (data not shown).

Building on this knowledge, heterozygous F1 plants were made by crossing 22.16 plants with wild type 2xCol plants. Due to the parent-of-origin effects observed on the size of seed resulting from these crosses, and the possibility of downstream effects on plant development, both reciprocal crosses were carried out.

To answer question 2, both sets of F1 plants were analysed for their floral phenotype. All the F1 plants tested showed full fertility suggesting that the reduced fertility phenotype is a recessive trait. However, no measurements were made on the length of the stamens or gynociea of these F1 flowers.

To determine if the altered seed size was inherited as a dominant, semi dominant or recessive trait F2 seed were collected from both sets of F1 plants and measured for their mature mass. As the F2 seed were heterozygous for the mutation, we could also study if the altered seed phenotype depended on the genotype of the gametophyte or the sporophyte (the maternal parent). Half of the F2 seed inherited the 22.16 mutation, therefore were the altered seed phenotype dependent on the genotype of the ovule then 50% of the seed would express the mutant phenotype. However, were the mutation to have a sporophytic effect then all the F2 seed should have a uniform phenotype.

The results are summarised in Figure 7.22. The F2 seed from both [$22.16^{maternal}$ x 2xCol] and a [2xCol x $22.16^{paternal}$] F1 plants were uniform in size, suggesting that the altered seed phenotype depends on the genotype of the maternal parent. However, the 2 F1 plant lines gave seed with different average mature mass. If the seed had a 22.16 grandmaternal parent (Figure 7.22 B) they were 32 % larger than seed that had a wild type grandmother.

In summary, preliminary genetic data suggests that both the reduced fertility phenotype and altered seed size of the line 22.16 line are closely linked and could be the result of the same mutation. The initial results suggested that the floral phenotype is recessive. However, it is possible that the elongation of the gynocieum is expressed as a semi-dominant trait, but that this intermediate degree of elongation is not sufficient to cause the reduced fertility phenotype.

The altered seed phenotype is expressed dependent on the genotype of the maternal parent, but only if the 22.16 mutation was inherited via the maternal grandparent. As the size of F2 seed from a [$22.16^{maternal}$ X 2xCol] F1 parent is intermediate between that of seed from a 22.16 and a 2xCol plant, this suggests that the altered seed phenotype is expressed as a semi dominant trait.

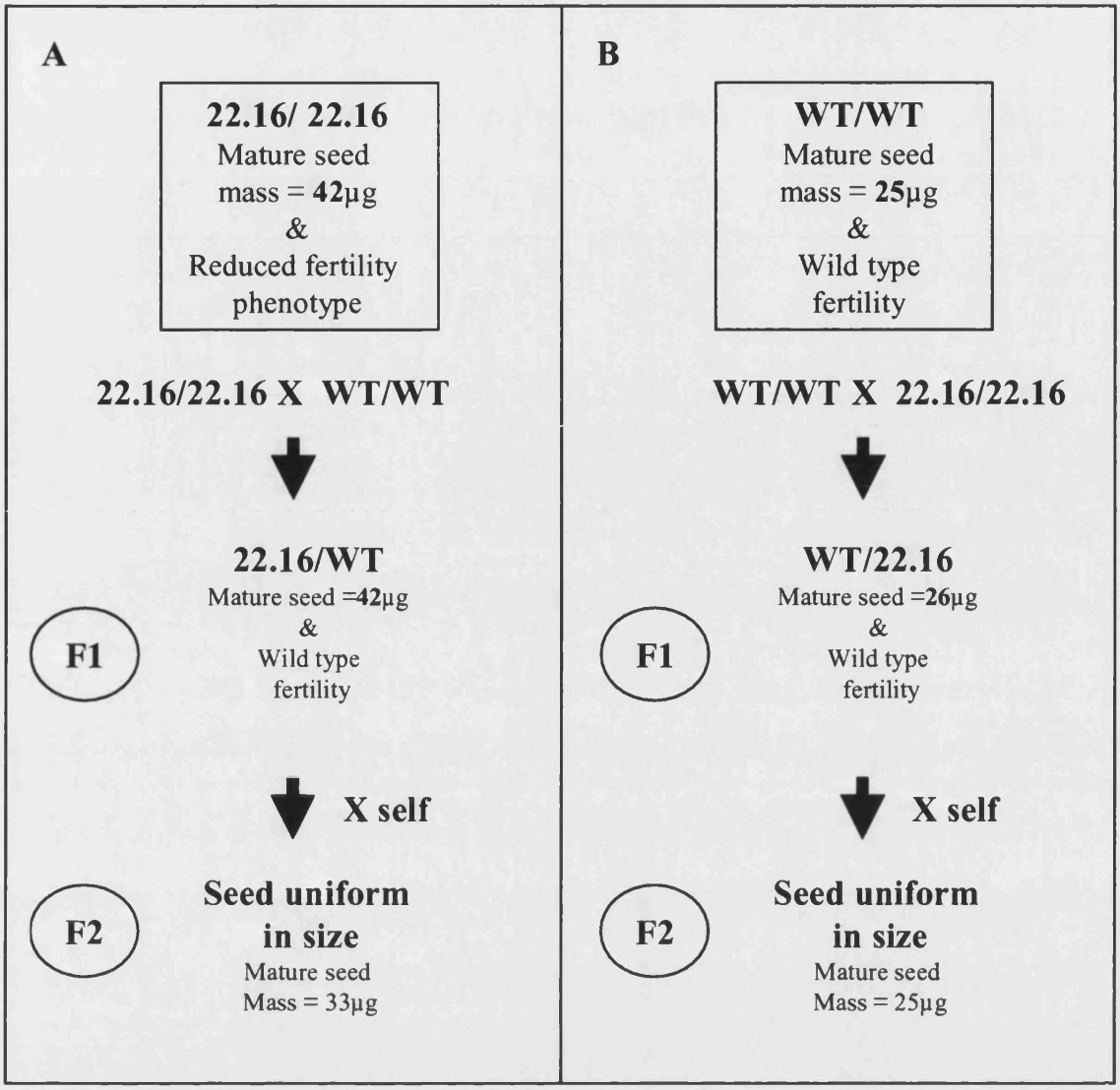


Figure 7.22

The grandmaternal effect on seed size in the 22.16 candidate line.When the F1 plant inherited the 22.16 locus from the maternal parent it produced F2 seed with a mass intermediate between that of 22.16 and wild type (WT) 2xCol seed (A). In contrast when the F1 plant inherited the 22.16 locus from the paternal parent the F2 seed had a mass comparable to WT (B). In both crosses there was only one class of F2 seed size suggesting that the expression of the altered seed phenotype depends upon the genotype of the maternal parent, and not that of the gametophyte.

7.3 Discussion

The mechanisms behind the phenomenon of genomic imprinting in plants remain elusive. Although DNA methylation has been attributed a global role in the maintenance of the imprinted state, how alleles are sex-specifically modified is unknown. Furthermore, only a few imprinted genes have actually been identified in plants. Therefore the objective was to design and carry out a screen for genes involved in the imprinting system, with the aim of providing a greater insight into this intriguing epigenetic process.

7.3.1 The screen successfully isolated maternal and paternal excess candidates from an EMS mutagenised population

As described in detail in section 7.1.2 (Figures 7.1 and 7.2) the screen was designed on the model that a mutation in a component of the imprinting system could be expressed as an alteration in seed size, based on the previous observations of seed development in interploidy crosses (Scott et al., 1998) and crosses between hypomethylated and wild type plants (Chapter 3). For example, a null mutation in a gene required for silencing endosperm-promoting genes in the maternal genome could result in a seed with an over proliferated endosperm and a corresponding increase in mature seed size. As the component of the imprinting machinery is sex specific (for example required for maternal silencing) the seed phenotype will only be expressed in a parent-of-origin dependent manner.

A 2 step screen was designed. The first step isolated seed from an EMS mutagenised population that were either maternal (smaller in mass than a wild type 2x control) or a paternal excess candidates (larger than a wild type 2x control). The second part of the screen ensured that the altered seed phenotype was expressed in the next generation. The first step of the screen used a series of sieves with different mesh sizes to grade seed according to volume. The sieving method used was efficient, with a positive correlation observed between mesh size and seed weight. Furthermore, seed from a paternal excess [2x X 4x] and a maternal excess cross [4x X 2x] was retained in sieve sizes, which retained only a very small percentage of wild type 2x seed. The EMS treated seed population used in the screen also had a higher percentage of seed that

were retained in these 'maternal' and 'paternal' excess sieves, than a wild type population. Hence the method of sieving an EMS treated population to isolate seed with a mass larger, or smaller, than a wild type control had good prospects for identifying candidate mutants in the genomic imprinting system. Furthermore, the innate simplicity of the sieving method enabled the screening of 77 000 EMS treated seed for an alteration in mass in just a matter of hours.

A large amount of candidates were isolated by the sieving method. Unfortunately the rate of germination was very low for the maternal excess candidates. There may be a number of reasons for the poor viability of seed isolated in this section of the screen. It was anticipated that many 'small' seed would be isolated in the screen that had mutations in genes that were not involved in genomic imprinting. For example, detrimental mutations in metabolic, cell cycle or growth promoting pathways, to name but a few, could result in small seed. Also, were these mutations having a drastic effect on seed development and resulting in abortion, then these seed will also be isolated in the 'maternal excess' sieves due to their comparatively small size.

Therefore to improve this part of the screen, potentially aborted seed might be identified by eye and removed from the candidate population. This however introduces a level of subjectivity to the screen. In preference, all the seed isolated in the maternal excess-like screen should be sown (perhaps in large trays) and the resulting seedlings then transferred to the larger pots, thus saving a large amount of space and maximising the prospect of isolating mutants in genomic imprinting. In contrast, the screen for paternal excess candidates that expressed the altered seed phenotype in the next generation proved more successful. Of the 141 plants tested, 18 (12.8%), were confirmed as maintaining a mature seed mass larger than the 2xCol control.

7.3.2 The ecotype C24 produces larger seed and this trait is expressed in a parent-of-origin manner in crosses with other ecotypes

According to the model (Figure 7.1), were the altered (large) seed phenotype of the paternal excess candidates a result of a mutation in the sex specific imprinting machinery, the seed phenotype should be expressed only when the mutation is

inherited through the maternal parent in reciprocal crosses with wild type plants. These crosses were originally conducted with 2xC24 plants, as a male sterile transgenic line was available (Paul et al., 1992). However, the C24 ecotype produces larger seed than the 2xCol ecotype (36 μg compared to 22μg). Unknown to us at the time the experiments were designed, this larger seed size of the C24 ecotype can be inherited in a parent-of-origin dependent manner (through the maternal parent) in crosses with other ecotypes (Alonso-Blanco and Koornneff, 2000). Therefore this was probably masking any parent-of-origin effect on seed size from the candidate lines in these crosses. The crosses with 2xCol as the wild type parent were then conducted with the candidate line 22.16 with some exciting results.

7.3.3 Characterisation of the paternal excess candidate line 22.16

7.3.3.1 The development of the endosperm is abnormal in the paternal excess candidate line 22.16

Preliminary characterisation of the candidate line 22.16 showed it to fit many of requirements predicted by the model for mutants in the maternal imprinting system (Figure 7.1). The plants produced seed that had a large seed mass in comparison to a wild type 2xCol control and the altered seed phenotype was only expressed when the mutant line was used as the maternal parent. Building on these exciting observations, further detailed characterisation of the candidate line was carried out. One of the primary predictions of the model is that if the altered seed phenotype is due to a paternal excess imbalance of imprinted gene expression, then the mutant seed should show an increase in endosperm proliferation in comparison to a wild type control. The results from the study of endosperm development in the mutant line 22.16 were not only unexpected with respect to the model, but also for seed development as a whole.

The development of the CE of 22.16 differed from that of the wild type equivalent. The CE of 22.16 was longer and thinner than the characteristic domed shape, observed in wild type seed. It is conceivable that this distortion of CE shape was due to the elongation of the adaxial ridge, observed in all 22.16 seed. However, in many cases the CE also showed other morphological abnormalities, including diffuse cytoplasm and the presence of unusual fibrous structure. Particularly in the later

stages of development (6 to 7DAP) the CE of 22.16 seed also showed little direct association with the CPT. Combined this suggests that the mutant 22.16 seed are exhibiting defects in the control of CE development.

Intriguingly, the abnormal development of the CE in the line 22.16 appears to contradict the proposed function of this structure. The CE is thought to play an important role in the uptake and processing of maternal resources for the developing seed (Nguyen et al., 2000). Yet in most 22.16 seed the CE had diffuse cytoplasm and little direct contact with the maternal tissue. The dense cytoplasm contains the ER, dictyosomes, nuclei, plastids and mitochondria, which are thought to process the nutrients and provide the energy for their transport into the developing seed (Nguyen et al., 2000). In wild type seed the intimate contact between the CE and the CPT, often maximised by feet like protrusions of the CE into the maternal tissue, suggests a role for the CE in facilitating the transfer of maternal resources into the seed. Hence defects in CE development would be predicted to have a detrimental effect on seed development, perhaps reflected by a smaller mature seed size. However 22.16 seed have a larger mature seed size compared to wild type 2xCol seed.

One interpretation of these observations is that the role of the CE in seed development may have been wrongly assigned. Alternatively, the poor CE development in 22.16 seed may be compensated for by the enhanced development of a different 'nutrient transfer' pathway. A third possibility is that the comparatively large mature seed phenotype of the 22.16 line is due solely to an increased proliferation of the maternal tissue, as opposed to enhanced endosperm and embryo development. However, a mature 22.16 embryo is larger in size than a wild type 2xCol mature embryo, suggesting that the larger seed size is not simply due to a heavier seed coat. It is possible that an increased growth of the maternal tissues could have an indirect effect on embryo size. If, for example, the volume of the central cell limits embryo growth, a larger central cell could promote a corresponding increase in embryo size.

The abnormal CE development of 22.16 seed does not directly support the hypothesis that the altered seed phenotype of this candidate line is the result of a paternal excess imbalance in imprinting, resulting in an increase in endosperm proliferation. Seed resulting from a paternal excess cross, for example a [2xC24 X 4xC24] cross, show

increased proliferation of the CE and a larger number of chalazal nodules compared to seed from a balanced [2xC24 X 2xC24] cross. However 22.16 seed produced no more chalazal nodules than the equivalent wild type control. Due to the distortion of the chalazal pole it was very difficult to deduce if the actual chalazal cyst in 22.16 seed had an increased volume compared to the wild type. Collectively the evidence suggests that the altered seed phenotype of the candidate line 22.16 did not arise due to an increased proliferation of the CE.

The analysis of the development of the PE in 22.16 also proved difficult for a number of reasons. If the mutant line phenocopied the PE development of a paternal excess cross, then the PE would have a larger number of nuclei, which cellularized later than a wild type seed. However, development of the PE in 22.16 seed appeared comparable to that in the balanced [2xCol X 2xCol] cross, with both cellularizing at the heart stage of embryo development. Yet, the mutant line often showed a delay in embryo development when compared to a wild type 2xCol seed. Such a delay in embryo development may not be reflected in the rate of division of the PE nuclei, and thus at the point of cellularization the mutant seed may have had more PE nuclei. Unfortunately the number of PE nuclei in the 22.16 seed at the point of cellularization could not be counted, due to the unusual structure of the mutant seed.

Detailed study of the mutant seed after PE cellularization showed an extremely unusual phenotype. No cellularized endosperm could be observed after 6 to 7 DAP. However, throughout the seed large nucleate structures could be observed which often appeared as separate entities, surrounded by a compact sphere of cytoplasmic material (Figure 7.13). No such abnormalities were observed in the wild type seed or had been reported for previous interploidy crosses in *A.thaliana* (Scott et al., 1998). Although little is known about the formation, or origin, of these large nucleate objects, similar structures have been described in the *titan* (*ttn*) class of mutants, in *A.thaliana*.

The *ttn* mutants exhibit dramatic alterations in mitosis and cell cycle control during seed development and are characterised by the formation of giant polyploid endosperm nuclei, with enlarged nucleoli (Liu and Meinke, 1998; McElver et al., 2000). It is these giant nuclei that resemble the nucleate structures of 22.16. The 22.16 candidate line shows the greatest phenotypic similarity to the *ttn3* mutants (Liu and

Meinke, 1998). The *ttn3* seed exhibit the characteristic large nuclei, yet produce viable seed, unlike many *ttn* mutants, where the embryo arrests shortly after fertilization. The *TTN3* gene encodes a protein related to the SMC2 family of condensins (McElver et al., 2000). These proteins are required for chromatin condensation, in yeast and animal systems and could therefore be required for normal chromosome mechanics at mitosis (Hirano, 1999). Another *TTN* gene, *TTN5*, has been shown to encode a protein related to the ADP ribosylation factor (ARF) family of small GTP binding proteins (McElver et al., 2000). Although *TT5* is most closely related to the ARL2 subfamily of ARF-like proteins (ARL), whose function are unclear, ARF proteins are known to have important roles in vesicle transport and signal transduction pathways (Martin, 1998; Chavier and Goud, 1999; Godi et al., 1999). Models for the role of TT5 include, involvement in the distribution of Golgi-derived materials required for the progression of mitosis or as a player in signal transduction pathways regulating cytoskeletal organisation. (McElver et al., 2000). Therefore the large nucleate structures observed in the central cell of the 22.16 mutant seed suggests that these seed may be defective in mitosis or cell cycle regulation.

In summary, the candidate line 22.16 produced seed with abnormal endosperm development. According to the model (Figure 7.1), were the altered seed phenotype of 22.16 a result of a mutation in the maternal genomic imprinting machinery the mutant seed should show an increased proliferation of the endosperm. Instead the candidate line produced seed with an abnormal CE that later in development showed little association with the maternal CPT. The abnormal shape of the mutant seed complicated the analysis of PE proliferation. However the production of large nucleate structures in the 22.16 seed, combined with the abnormal development of the CE, suggests that the wild type 22.16 product may have a role in the regulation of the cell cycle. This proposed function is also in accordance with the increased number of cells in the inner integument and the elongation of the gynocieum and will be discussed in detail later.

7.3.3.2 Seed from a [22.16 X 2xCol] cross have a larger CE than either of the parents

The model on which the screen was based (Figure 7.1) predicted that if the abnormal endosperm development, observed in the candidate line 22.16, was indeed due to a mutation in maternal component of the imprinting system, this phenotype should be expressed only when the mutation was inherited from the maternal parent. Indeed, the seed from reciprocal crosses between 22.16 and 2xCol showed different CE phenotypes which were in accordance with the model. The development of the CE in [2xCol X 22.16] seed was comparable to the wild type control. In contrast, seed from a [22.16 X 2xCol] cross, produced an elongated CE that had a much larger volume than the wild type. Intriguingly, the CE of the [22.16 X 2xCol] seed was also larger than the CE of [22.16 X 22.16] seed, suggesting that the wild type 22.16 locus provided by the paternal 2xCol parent allows greater proliferation of the CE. Furthermore, the [22.16 X 2xCol] seed showed none of the nucleate structures described for the [22.16 X 22.16] seed. Despite the differences in endosperm development both the [22.16 X 22.16] and the [22.16 X 2xCol] cross produce seed with similar average mature mass. Could this indicate an uncoupling of endosperm and embryo development?

One possible explanation for the observed difference is that the maternal 22.16mutation could cause a defect in cell cycle regulation, allowing the greater proliferation of the CE. In [22.16 X 2xCol] seed the wild type 22.16 locus may be initially silenced in early development. Indeed, Vielle-Calzada et al., (2000) predicted that the majority of the paternal genome might be silenced in the developing seed until 80 HAP. Thus only the maternally inherited mutant 22.16 product would be expressed, allowing the increased proliferation of the CE. Later in development, the paternally inherited wild type 22.16 locus would be active, allowing wild type cell cycle progression. The larger CE could therefore develop from the greater number of cells laid down prior to the activation of the paternal genome. In contrast, [22.16 X 22.16] seed have no wild type 22.16 locus and this might result in an increased disruption of the cell cycle, accumulating in the abnormal CE and large nucleate structures.

7.3.3.3 22.16 ovules are larger in size than the wild type control

The ovules of a 22.16 plant were larger in size than wild type 2xCol ovules (Figure 7.19), suggesting that the wild type 22.16 product could act to limit ovule development prior to fertilization. If, for example, the 22.16 wild type product silences growth promoting paternal genes in the maternal genome, in the candidate line these genes may be ectopically expressed in the ovule, resulting in the increased size. Indeed, some mutant alleles of the *FIS* complex genes, *MEA*, *FIE* and *FIS2*, confer a degree of autonomous endosperm development on unfertilized ovules (Ohad et al.,1996; Chaudhury et al.,1997; Ohad et al.,1999; Kiyosue et al.,1999; Luo et al.,1999). One proposal is that this phenotype results from the release of repression of endosperm-promoting genes from the central cell nuclei (Yadegari et al., 2000; Vinkenoog et al., 2001). However, it should be noted that no increase in size has been reported in unfertilized *mea*, *fie* or *fis2* ovules. Furthermore, the 22.16 ovules showed no autonomous development of the endosperm to correlate with the increase in size. Alternatively, the difference in ovule size could be the result of defects in regulatory factors that do not function in genomic imprinting. The wild type 22.16 product may act to limit growth, perhaps by the inhibition of the cell cycle.

7.3.3.4 Seed with a 22.16 maternal parent are longer, with more cells in the inner integument compared to those with a wild type maternal parent

At the globular stage of development, seed that had a 22.16 maternal parent were 50% longer and 25% wider than seed with a wild type maternal parent. This difference in seed length correlated with an increase in the number of cells in the inner integument. Magnification of cells from the four seed types (Figure 7.20 C), showed them to have comparable dimensions, suggesting that the seed elongation observed when 22.16 is used as the maternal parent is due to an increase in cell number, as opposed to increased cell expansion. Once again this is consistent with the proposal that the wild type 22.16 product has a role in cell cycle control.

This does not directly contradict the model for the altered seed size resulting from a defect in genomic imprinting. Seed from viable paternal excess crosses (for example [2xC24 X 4xC24]) are larger than 2x seed (Scott et al., 1998) and this must have

occurred due to an increase in cell division. However, unlike paternal excess crosses, the endosperm of [22.16 X 22.16] seed does not appear to have proliferated to a greater extent than in the wild type control. Therefore, we may cautiously state that the increase in size is due to a direct increase in cell division within the seed integuments, as opposed to an increase in division stimulated by greater proliferation of the endosperm.

7.3.3.5 The floral organs are elongated in 22.16 plants

Perhaps the most striking phenotype of the candidate line was the reduced fertility most likely caused by the elongation of the floral organs. At the later stage of floral development (when the flowers had been open for a period of 2 days) the gynocieum of a 22.16 flower was 25% longer than the wild type equivalent. Although no detailed cellular analysis was carried out on the floral organs, the elongation of the structures suggests an increase in cell number or cell expansion. Given the previous results for the elongation of the seeds with a maternal 22.16 parent, we can tentatively predict that the elongation seen in the gynocieum was also due to an increase in cell number.

Interestingly, the stamens of the 22.16 flowers showed only a minor increase in length in comparison to the elongation of the gynocieum, suggesting that the 22.16 mutation is specifically effecting the female floral organ. As the 22.16 ovules also showed an increase in size, this could indicate that the wild type 22.16 product acts specifically on cell cycle regulation in the maternal reproductive organs. Alternatively, the increased number of cells in the gynocieum wall could signal to the ovules, or visa versa, stimulating the other to proliferate.

7.3.3.6 The genetics of the 22.16 mutation

Evidence suggests that the original 22.16 candidate plant was homozygous for the mutation/s that gave rise to the floral and seed phenotypes. It is also probable that these traits, as the preliminary results suggest that they are closely linked and they share a common theme of elongation, are the result of the same mutation.

The altered seed phenotype was expressed in a semi-dominant manner, depending on the genotype of the maternal parent. Considering that genomic imprinting in plants is thought to primarily target the endosperm, one might logically expect that the expression of the mutant phenotype would be dependent on the genotype of the gametophyte (polar nuclei). Indeed, the lethal phenotype of seeds that inherit a maternal *mea* allele (*MEA* is the only known imprinted gene in *A.thaliana*) depends on the genotype of the ovule and not the maternal parent (Peacock et al., 1995; Chaudhury et al., 1997; Grossniklaus et al., 1998). This suggests that the wild type 22.16 product might not play a part in genomic imprinting. However, as so little is known about the mechanism of genomic imprinting, we would be foolish to dismiss such a role for 22.16 without further analysis.

Perhaps the most intriguing aspect of 22.16 is the grandparent-of-origin dependent expression of the altered seed phenotype (Figure 7.22 and 7.23). The 2 sets of F1 plants (A and B) both carried one 22.16 and one wild type allele and only differed in the parental origin of the 22.16 mutant locus. One possible explanation for this is that the 22.16 allele is modified when it passes through the paternal germline, and once modified the allele loses the altered seed size activity. Such parental specific modification of the allele would suggest that 22.16 is a target for genomic imprinting. However, the 22.16 plants have been inbred for a number of generations and all the progeny produced seed with the altered seed phenotype (data not shown). If the mutation was reset by passing through the male germ-line then the altered seed phenotype would have been diluted with each generation, as 50% of the 22.16 loci must have been inherited through the paternal parent. This would not be the case were the mutant maternal 22.16 allele able to influence the paternal allele in the sporophyte, if the paternal allele was also mutated. We could envisage a situation similar to paramutation (reviewed in Chandler et al., 2000), In paramutation 2 alleles interact in the heterozygote leading to the stable, though reversible, epigenetic silencing of one of the alleles. In 22.16 this interaction may only occur in the maternal parent due to sex-specific as yet unidentified factors.

7.3.3.7 The possible function of the wild type 22.16 product

Strikingly, many of the pleiotropic phenotypes observed in the mutant line 22.16 share a common theme. The ovules, developing seed and gynociea were all larger in size than a wild type control. Furthermore, the alteration in size often resulted in the elongation of the affected structure. Analysis of the integuments of a 22.16 seed showed that the increase in seed length was due to an increase in the number of cells, as opposed to cell expansion. Although the gynociea and ovules have not yet been studied at the cellular level, the phenotypes suggest that the candidate line carries a defect in cell cycle regulation.

The plant cell cycle is under the control of many genetic and environmental factors. Progression through the cell cycle is driven by heterodimeric protein kinases. These kinases consist of a catalytic subunit, termed cyclin-dependent kinases (CDK), and an activating subunit, cyclin (reviewed in Mironov et al., 1999; den Boer and Murray, 2000). The active kinase complex can trigger specific gene expression. For example, the CDK-cyclin complex phosphorylates the retinoblastoma pRb protein, releasing the E2F transcription factor from repression and allowing the expression of S-phase genes. The kinase proteins are themsleves regulated at a number of levels.

All CDKs require phosphorylation of a conserved thronine residue for activation, whilst the phosphorylation of specific theronine (T14) and/or tyrosine (Y15) residues inactivates the CDK. The CDK inhibitors (CKIs) provide a further level of regulation by binding either the cyclin, the CDK, or the CDK-cyclin complex and inhibiting their association.

Theoretically an increase in cyclin or CDK production could result in the increase in cell proliferation observed in the line 22.16. Indeed, the expression of the mitotic cyclin CycB1 under the control of the widely expressed cdc2a (CDK-a) promoter, resulted in an increased growth rate in the root (Doerner et al., 1996). As with 22.16, the increase was primarily due to an increase in cell number rather than cell size. Interestingly, most experiments either reducing or increasing CDK-a activity have shown it to have little effect on cell division, suggesting that this group of CDKs at least are not a limiting factor in plant cell division (reviewed in den Boer and Murray,

2000). Hence the altered seed and floral phenotypes could be caused by a GOF mutation in a cyclin or a CDK that acts specifically during the development of these structures.

The up regulation of cyclin-CDK activity could also occur via a LOF mutation in a negative regulator of the cell cycle, for example in one of the ICKs or an enzyme responsible for the activating phosphorylation of the theronine residue. Over expression of ICK1 in 35S-ICK1 transgenic plants resulted in plants that weighed less than 10% of the wild type after 3 weeks of growth (Wang et al., 2000). This reduction in size correlated with a reduced number of cells. Therefore a lower concentration or reduced activity of an ICK could theoretically result in an increase in cell proliferation. Hence the 22.16 mutation could effect the expression of a gene whose function is to negatively regulate cell cycle progression. Such an inhibitor may have a direct action, such as an ICK or an indirect action such as the *Clavata* (*CLV*) loci. *CLV 1*, *CLV2* and *CLV3* encode proteins that are involved in signal transduction pathway promoting the progression of peripheral stem cell daughters toward organ initiation (Clark et al., 1997; Fletcher et al., 1999; Jeong et al., 1999). Mutation in these proteins causes a delay in this progression, resulting in an accumulation of stem cells and a subsequent increase in size of the stem apical meristem (SAM) and floral meristem (FM) (Clark et al., 1993, 1995; Kayes and Clark, 1998; Laufs et al., 1998).

The endosperm phenotype observed in 22.16 seed also points to additional ways in which the 22.16 mutation could be affecting cell cycle regulation. As discussed previously the nucleate structures in the 22.16 seed closely resemble the giant polyploid nuclei characteristic of the *ttn* class of mutants (Liu and Meinke, 1998; McElver et al., 2000). The cloned *TTN* genes have predicted roles as diverse as the condensation of chromosomes, vesicle transport and signal transduction. This illustrates the huge variety of target genes in which defects can result in the misregulation of cell division observed in the 22.16 mutant line.

Another striking aspect of the altered seed phenotype seen in seeds with a maternal 22.16 parent is the conservation of the phenotype over all the progeny, even if they inherit a wild type paternal allele. The evidence described in this Chapter suggests that the enlarged seed phenotype is due to an increase in cell number (and by

inference an increase in cell division) and that this increase is 'controlled' with [22.16 X 22.16] and a [22.16 X 2xCol] seed showing a similar numbers of cells in the integuments. This could suggest that that the 22.16 product either limits or stimulates cell division for only a short developmental period. Alternatively, other factors may act to limit the degree of proliferation, preventing the cells dividing out of control.

7.3.4 Future work

The work concerning both the sieving screen for mutations in components of the genomic imprinting machinery and the characterisation of the candidate line 22.16 are still in their infant stages, however the preliminary data described in this Chapter is extremely encouraging. At the point of writing mapping experiments are underway to isolate the affected genomic region in 22.16. The sieving screen is also currently being repeated (including the modifications listed in Section 7.3.1) and other candidates from the first screen are being characterized.

7.3.5 The variation in seed development in interploidy crosses with different ecotypes

7.3.5.1 The ecotype Col shows different endosperm and seed development to C24 in paternal excess crosses

The interploidy crosses carried out using the ecotype Col gave highly unexpected results, particularly within the control crosses. The maternal excess cross [4xCol X 2xCol] gave a high proportion of viable seed with a small mass and an under proliferated endosperm. These phenotypes were similar to those previously reported for the equivalent crosses in the ecotype C24 (Scott et al., 1998). In contrast, the development of seed from the paternal excess cross [2xCol X 4xCol] differed dramatically from that published for C24. Whilst a [2xC24 X 4xC24] cross produced large viable seed, with a highly proliferated endosperm (Scott et al., 1998 and Figures 3.7, 3.8) seed from a [2xCol X 4xCol] cross show a low viability (29.6%) with no distinct enlargement of the CE, or over proliferation of the PE. [2xCol X 4xCol] seed also exhibited abnormal PE development, such as the strings of distorted PE nuclei,

which had been previously observed in seed from the lethal extreme paternal excess cross [2xC24 X 6xCol] (Scott et al., 1998). Furthermore, the CE also showed little direct contact with the CPT at later stages of seed development. Combined this suggests that the high rate of seed abortion from the [2xCol X 4xCol] cross was due to abnormal endosperm development.

The inter-ecotype crosses also showed some interesting results for the paternal excess crosses. If the paternal parent in was replaced with one of the C24 ecotype, [2xCol X 4xC24], the lethal phenotype of the Col paternal excess cross was rescued completely. However, if the maternal parent was replaced, [2xC24 X 4xCol], the rescue of lethality was not as dramatic. Unfortunately, time restraints meant the development of the endosperm could not be examined in these crosses.

Thus the ecotypes C24 and Col differ in their ability to produce viable seed from a paternal excess cross that results in a 2m:2p ratio of parental genomes in the endosperm. One possible explanation for this observation is based on the model of genomic strength (or Endosperm Balance Number (EBN)) (Ehlenfeldt and Ortiz, 1995). As described in Section 1.5 this theory gives each species a genome specific effective ploidy level, its EBN, which may not be equivalent to its actual ploidy level. In an interspecific cross it is the EBN balance that must be in a 2m:1p ratio to allow the correct development of the endosperm. The higher the EBN value the higher the genomic strength of the species. A higher genomic strength could be speculated to be due to a number of factors including, more genes in total subjected to imprinting, a larger number of genes with a greater effect on seed/endosperm development targeted by the imprinting system, tighter control of gene silencing or a longer period uniparental gene expression. If the model of genomic strength is extended to different ecotypes, which may have accumulated genetic differences due to geographical separation, Col could be seen to have a higher genomic strength than C24.

The C24 ecotype has been reported as a modifier of imprinting, with respect to the *MEA* locus (Vielle-Calzada et al., 1999; Kinoshita et al., 1999; Luo et al., 2000). Theoretically when a plant homozygous for a mutation in MEA, *mea/mea*, is pollinated with wild type pollen no viable seed should be produced as all inherit a

maternally derived *mea* allele. However, Luo et al., (2000) reported that when the wild type pollen is of the C24 ecotype, 24% of the seed produced are viable.

Could this model of genomic strength explain the differences observed in the inter-ecotype paternal excess crosses? If the C24 ecotype is used as the paternal 4x parent [2xCol X 4xC24] the lethality of the Col paternal excess cross was rescued to a much greater extent than when C24 is used as the maternal parent [2xC24 X 4xCol]. Were the genomic strength of Col greater than that of C24, this would be more apparent in the latter cross as the 4x Col parent is contributing twice as much genetic material. However, the ecotype effect on seed development is not reciprocal, in that the maternal excess crosses, [4x X 2x] regardless of which combination of parental ecotypes were used, gave seed with similar phenotypes. The explanation could lie in the evolution of imprinting.

As described in Section 1.3.4 *A.thaliana* is a predominantly self-fertilizing species, which might have retained the imprinting system from an out-crossing ancestor (Stebbins, 1974; Scott et al., 1998). Furthermore, it was proposed that the imprinting system of *A.thaliana* has broken down over time and that this has allowed the tolerance of imprinting imbalance in the endosperm observed in the C24 ecotype crosses (Scott et al., 1998). If the imprinting system of the ecotypes under study had been exposed to different selection pressures the systems could have broken down to different degrees, resulting in the variation in genomic strength. Also, within an ecotype the maternal and paternal imprinting systems could have been under different selection pressures. For example, in Col the paternal imprinting system may have been under greater selection pressure, so that the paternal genomic strength was maintained more than that of the reciprocal maternal system. Our results are not an isolated example. Indeed, a previous report by Rédei (1964) on interploidy crosses in *A.thaliana* also reported that [4xX 2x] crosses were viable, whilst the reciprocal cross produced aborted seed. Unfortunately the author did not state the ecotype used in the crosses, although it is likely that it was the Col ecotype. A preferential intolerance to a paternal excess imbalance has also been reported for a number of species including maize (reviewed in Vinkenoog et al., 2002).

So why would there be a stronger selection pressure against more paternal genomes in the endosperm? The paternal excess crosses in C24 led to an over proliferation of the endosperm and a subsequent increase in seed size. If nutrients were scarce this may have selected against the tolerance of extra paternal genomes (and thus extra endosperm-promoting genes), acting as a brake on seed development. In support of this theory the 2xCol seed were significantly smaller than 2xC24 seed (22μg compared to 36μg) despite the fact that were grown under the same conditions, suggesting that the Col ecotype does limit seed size to a greater extent.

However, it should be considered that the differences observed might be due to factors other than imprinting. A large proportion of seed from a [2xCol X 4xCol] cross aborted at the globular stage of development, with little evidence of over proliferation of the CE or PE endosperm. This is in direct contradiction with the phenotype of seed from the extreme paternal excess cross [2x X 6x], which showed an extreme over development of the CE and PE, prior to embryo abortion (Scott et al., 1998). An interesting point to note is that the 6x plant used in these experiments was actually of the Col ecotype (with a 2x C24 maternal plant). Thus the seed from a [2xCol X 4xCol] cross could carry the potential to produce a large over proliferated endosperm, but the seed die before the realization of this potential. The future study of CE and PE endosperm development in seed from a [2xC24 X 4xCol] and a [2xCol X 4xC24] could shed light on this mystery.

A further point to note is the potential of genomic alterations that could have occurred in the 4x plants. Recent studies on colchicine induced 4x *A.thaliana* plants showed a chromosomal rearrangement of the 45S rRNA locus when compared to 2x plants of the same ecotype (Weiss et al., 2000). If genomic alterations have occurred in 4xCol or 4xC24 plants this could affect gene expression due to the actual mutation of genes or by epigenetic changes. For example, euchromatic regions could become adjacent to heterochromatic regions, leading to changes in the higher order chromatin structure. This could affect the imprinting system directly, or affect development so that when the plant is under additional stress caused by the paternal excess imbalance the seed abort.

Expanding on the potential effect of genomic alterations, a number of orthologous genes have been shown to be epigentically silenced in polyploid species. *Arabidopsis suecica* is a natural allotetraploid that is formed from by the interspecific hybridization between *A.thaliana* and *Cardaminopsis arenosa.* 10 genes with a variety of functions are epigentically silenced from either *A.thaliana* or *Cardaminopsis arenosa* in *A.suecica* (Lee and Chen, 2001). Similar silencing could occur in the 4xC24 and/or 4xCol autotetraploids and this could effect gene expression, which could then affect the tolerance of imprinting imbalance.

In conclusion, the C24 and Col ecotypes differ in their ability to tolerate a paternal excess imbalance in the endosperm of developing seed. A number of alternative theories have been outlined but due to the lack of available data these are highly speculative. Further detailed analysis of the 2 ecotypes, particularly with respect to endosperm development and the modifying effect of C24, could shed light on these ecotype differences. Such work may not only provide us with answers to this intriguing puzzle but also provide us with tools with which to study the mechanism and evolution of imprinting in *A.thaliana.*

7.3.5.2 The paternal excess cross [22.16 X 4xC24] produced larger seed with a higher rate of abortion than the [2xCol X 4xC24] cross

The original aim of these interploidy crosses was to study endosperm development in 22.16 interploidy crosses in comparison to control crosses using the Col ecotype. According to the model (Figure 7.1) were the 22.16 mutation resulting in a paternal excess imbalance in the endosperm, then the endosperm of [22.16 X 4xCol] should be proliferated to a greater extent than in the control cross [2xCol X 4xCol]. Furthermore, this extreme paternal excess imbalance could result in an increased rate of seed abortion (similar to the situation in a [2x X 6x] cross) (Scott et al., 1998). Although the unexpected results described above have complicated the analysis of these crosses a number of interesting observations were made.

When the 22.16 line was used in maternal excess crosses with either 4xCol or 4xC24 the seed obtained were similar in morphology and size to that of seed from both the [4xCol X 2xCol] and [4xC24 X 2xC24] crosses. This highlighted the fact that the

altered seed phenotype of 22.16 is only expressed if the mutation is maternally inherited.

In contrast, seed development in a [22.16 X 4xCol] cross was similar to that in a [2xCol X 4xCol] cross. The development of the endosperm of [22.16 X 4xCol] seed was highly abnormal and the cross produced a high proportion of aborted seed (germination rate 16%). Combined this suggests that the lethality observed in the [22.16 X 4xCol] cross was due to an effect of the Col ecotype, as opposed to a extreme paternal excess imbalance of imprinting resulting from the 22.16 mutation (superimposed on the [2x X 4x] cross). However, the paternal excess crosses conducted between 4xC24 and 22.16 plants produced seed with interesting phenotypes compared to the [2xCol X 4xC24] control. The replacement of the 4x parent with that one of the C24 ecotype, [22.16 X 4xC24], resulted in a significant level of rescue of the lethal phenotype (germination rate of 83%). Intriguingly, these seed had a larger mass (52μg) and a lower viability than seed from the [2xCol X 4xC24] cross (44μg and 96% respectively). This increase in seed size and abortion rate could be interpreted as a greater proliferation of the endosperm caused by a more extreme paternal excess imbalance in the [22.16 X 4xC24] seed. Future analysis of PE and CE development in these seed would allow the testing of this theory.

7.3.6 Summary

Analysis of the 22.16 candidate line has so far been unable to determine whether the line carries a mutation in a component of the genomic imprinting system. However, the characteristic elongation of many structures in the 22.16 line, combined with the abnormal endosperm development, point to defects in cell cycle regulation. Not only would the identification and analysis of the genomic region affected in the mutant line shed light on the process of cell division, it could also provide us with an excellent tool with which to manipulate plant and seed size. Considering the many concerns regarding transgenic biotechnology, the ability to manipulate seed and plant development without the introduction of transgenic DNA could prove invaluable to future agriculture research. Furthermore, the increase in seed size, without an apparent corresponding increase in endosperm development, suggests factors other than

endosperm proliferation can limit seed growth. The study of these factors might allow us a greater understanding of seed development and provide us with new tools of agricultural significance.

In conclusion, the screen designed to identify mutants in the sex-specific genomic imprinting system isolated an interesting candidate line, 22.16. Although the role of the wild type 22.16 product in genomic imprinting is debatable and requires further study, the mutant line does exhibit a number of interesting abnormal phenotypes in seed and plant development. Hence, the screen still has extremely good prospects of isolating mutants in the genomic imprinting system via an alteration in seed size.

Chapter 8

Discussion

8.1 The role of MET1 DNA methylation in genomic imprinting in *A.thaliana*

The primary aim at the beginning of the work described in this Thesis was to uncover the role of DNA methylation in imprinting in *A.thaliana*. We showed that reciprocal crosses between wild type and hypomethylated (*MET1a/s*) plants have a strong and distinct parent-of-origin effect on endosperm and seed development. From this we concluded that methylation plays an important role in the parent-of-origin effects, and by inference imprinting, in plants.

Since the beginning of these experiments a number of reports have been published concerning the role of DNA methylation in the regulation of the *FIS* class genes (*MEA*, *FIS2* and *FIE*). As described in Section 1.3.5 all 3 loci are suspected to be paternally imprinted (Vielle-Calzada et al., 1999; Kinoshita et al., 1999; Luo et al., 2000), and pollination of *mea*, *fis 2* or *fie* mutant ovules (in the vast majority of cases) results in seed abortion (Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus et al., 1998). Vielle-Calzada et al., (1999) showed the rescue of *mea* seed that inherited 2 copies of a mutation in the *ddm1* locus (Vongs et al., 1993). However, rescue only occurred if the pollen parent contributed a wild type *MEA* allele (Vielle-Calzada et al., 1999). The authors concluded that the rescue of these seed was due to the reactivation of the normally silenced paternal *MEA* allele. These results are consistent with our model that attenuating DNA methylation results in the expression of normally imprinted genes (Figure 3.16).

However, a second group showed that both *mea* and *fis2* seeds could be rescued using hypomethylated *MET1a/s* pollen without the contribution of a wild type paternal allele (Luo et al., 2000). Additional analysis also showed that if the pollen used was from inbred *ddm1* lines, then mea could also be rescued without a wild type paternal allele (Grossniklaus et al., 2001). In the original experiments (Vielle-Calzada et al., 1999) the parental plants were heterozygous for the recessive *ddm1* mutation.

Therefore, the earliest time point at which hypomethylation of the paternal genome could have occurred is at the first meiotic division, when the wild type and mutant *ddm1* alleles were segregated. Furthermore, Luo et al., (2000) did not show any reactivation of paternally inherited *MEA* and *FIS2* promoter GUS transgenes in a *MET1a/s* background. Combined this suggests that the rescue of the *mea* and *fis2* mutations with hypomethylated pollen is not occurring via the reactivation of the paternal allele. Both groups have suggested that the activity of paternal genes other than *FIS2* and *MEA* could be resulting in the rescued phenotype.

So what role does DNA methylation play in genomic imprinting in plants? The current information suggests that DNA methylation directed by the action of MET1 and DDM1 does not regulate the paternal silencing of *MEA* or *FIS2*. However, as discussed many times during this thesis, MET1 is only 1 out of 9 putative DNA methyltransferases in the *A.thaliana* genome. It therefore remains a strong possibility that DNA methylation has a role in the silencing of *MEA* and *FIS2*, but that this modification is under the control of another DNA methyltransferase.

Interestingly, the rescue of *fie* seeds with hypomethylated (*MET1a/s*) pollen has been shown to require a wild type paternal allele (Vinkenoog et al., 2000; Luo et al., 2000). Although this may be because *FIE* has an essential action, it can not be ruled out that activation of the paternal allele is leading to the rescued phenotype, and therefore MET1 directed methylation could well be involved in the predicted paternal silencing of *FIE*.

So how could demethylation of the paternal genome rescue *mea*, *fis2* and *fie* seeds? To attempt to answer this question we should first consider the wild type role of the *FIS* complex genes. All 3 proteins are predicted to have a role in repressing endosperm development, as mutations in the encoding loci result in a degree of autonomous endosperm development in ovules. *MEA* and *FIE* encode polycomb (PcG) proteins and have been shown to interact via yeast 2-hybrid analysis (Luo et al., 2000; Spillane et al., 2000; Yadegari et al., 2000). In mammals and *D.melanogaster*, PcG proteins are known to form multimeric complexes that promote the silent state of repressed genes by changing the higher order chromatin structure (Pirrotta, 1997). FIS2, a putative transcription factor, has been shown not to interact with either MEA

or FIE (Luo et al., 1999). However, as mutations in all 3 genes results in a similar phenotype its is predicted that they could act in a protein complex to repress the expression of endosperm promoting genes (Grossniklaus et al., 2001).

fie ovules fertilized with wild type pollen abort with a phenotype very similar to seed from the extreme paternal excess cross [2x X 6x] (Scott et al., 1998; Vinkenoog et al., 2000). One possible explanation is that *fie* seed have an extreme paternal excess imbalance of imprinted gene expression, as FIE usually acts to repress endosperm-promoting genes from the maternal genome. In other words, FIE is part of the complex that represses at least a proportion of maternally imprinted genes. This proposal can be extrapolated to the other *FIS* complex genes, *MEA* and *FIS2*. If this is the case, hypomethylated pollen could rescue *mea*, *fis2* and *fie* seeds as it provides, according to our model (Figure 3.16), extra active endosperm-inhibiting alleles (which are normally paternally imprinted) and this readdresses the balance of imprinted gene expression to a point that supports the production of viable seed.

This begins to suggest that different plant imprinted genes could be under different control mechanisms. For example, one set could be regulated to a certain extent by MET1 catalyzed DNA methylation (possibly including *FIE*), whilst a second set (including *MEA* and *FIS2*) could be silenced by other epigenetic modifications. In support of this argument, in mammals it is becoming apparent that at least one imprinted locus (Caspary et al., 1998; Tanaka et al., 1999) does not require DNA methylation.

Another factor to consider is that the *MET1::GUS* construct was not expressed in the endosperm after 3 to 4DAP (Chapter 6). This is evidence to suggest that even if MET1 does have a role in catalyzing imprinting-associated methylation it will not play a part in maintaining these methylation patterns in the endosperm after the late globular-early heart stage of development. Such methylation patterns could be propagated by other DNA methyltransferases. Alternatively, a repressed state, perhaps initially set and propagated by MET1 in the gametes and early seed, could be maintained by other chromatin components (such as histone methylation and acetylation). A third possibility is that it is the DNA methylation status of the DNA coming from the somatic genome, which initiates the establishment of the imprinted

state in the germ line. In this scenario, DNA methylation could direct sex-specific chromatin remodeling factors to imprinted loci, resulting in the establishment of a stably repressed epigenetic state.

Thus the exact role of MET1 catalyzed DNA methylation in genomic imprinting in plants still remains elusive. The distinct parent-of-origin effect on seed development where MET1 catalyzed methylation is attenuated in one of the parental plants in a cross is evidence to support MET1 as having a role in the parent-of-origin effects, and by inference genomic imprinting in plants. Although paternally inherited *MEA* and *FIS2* promoter transgenes did not show reactivation in a *MET1a/s* hypomethylated background, this does not rule out the role of MET1 DNA methylation in the regulation of expression of other (as yet unknown) imprinted genes. Indeed, the maternal effect *mea, fis 2* and *fie* mutations were all rescued when hypomethylated pollen was used, suggesting the altered activity of paternal genes (perhaps via an altered imprinting state) could compensate for these mutations. Furthermore, DNA methylation catalyzed by enzymes other than MET1 could still be important for the regulation of *MEA* and *FIS2* imprinted gene expression.

In conclusion, there is a large body of evidence to support a role for MET1 in genomic imprinting in plants. However, it is increasingly apparent that MET1 is not the only player. To begin to unravel the mechanism of genomic imprinting in plants we must start to consider the role of other DNA methyltransferases and chromatin components.

8.2 The role of other DNA methyltransferases and chromatin components in genomic imprinting in plants

To date, the role of DNA methylation in genomic imprinting in plants has centered on DNA methylation regulated by MET1 and DDM1. However, the potential role of other DNA methyltransferases, and their possible interaction with other chromatin

remodeling proteins and components, is becoming evident. The evidence from the *DRM2::GUS* expression studies suggests that *DRM2* expression is localized to the suspensor at very early stages of development (1 DAP). This is not only exciting with respect to the study of the role and development of the suspensor, but also highlights the possibility that the suspensor could be a target for genomic imprinting. As described in Chapter 6 (Section 6.3.3.7) evidence suggests that the suspensor plays an active role in embryo development by synthesizing growth factors and acting as a conduit for nutrient transfer between the surrounding tissues and the embryo (Yeung, 1980; Ceccarelli et al., 1981; Cionini, 1987; Piaggesi et al., 1989; Ciavatta et al., 2001). Therefore, the parental genomes could influence embryo development in a parent-of-origin manner by regulating gene expression in the suspensor.

Also of interest is the possible role of the CMT enzymes in genomic imprinting in plants suggested by recent studies of chromodomain protein. The *D. melanogaster* chromodomain proteins, MOF and MSL-3, were found to bind non-coding RNA molecules (Akhtar et al., 2000). Combined with the observation that small RNA molecules, cleaved from double stranded RNA (dsRNA), can induce DNA methylation of homologous sequences in plants (Wassenegger et al., 1994; Jones et al., 1999; Wassenegger, 2000; Mette et al., 2000; Matzke et al., 2001a) it has been proposed that CMTs could mediate RNA directed silencing (Matzke et al., 2001b; Papa et al., 2001). The small RNAs could direct this methylation by interacting with the CMT proteins via the chromodomain, and targeting the enzyme to the homologous DNA.

A further proposed function of CMT proteins links DNA methylation with histone methylation. Recently (Jackson et al., 2002) showed that CMT3 interacts with an *A.thaliana* homologue of the heterochromatin protein 1 (HP1), which in turn interacts with methylated histones. Further to this the authors reported that loss of function alleles in *KRYPTONITE* , a histone H3 Lys 9 methyltransferase gene, resembled *CMT3* mutants, with reduced CpNpG methylation. The authors proposed that the CpNpG methylation of CMT3 is regulated by histone H3 Lys 9 methylation, by the interaction of CMT3 with methylated chromatin, perhaps with the DNA methylation acting to re-inforce the silent heterochromatic state. Here we could envisage that CMT proteins could act to add methylation on to imprinted (silent) alleles,

contributing an extra layer of modification and stabilising the imprinted epigenetic state.

Thus DNA methylation theoretically could have a complex role to play in genomic imprinting in plants. Different DNA methyltranferases might regulate the expression of different sets of imprinted genes. DNA Methylation itself could also act at a number of levels. For example, DNA methylation may be the actual imprinting mark, and/or act to further stabilize an already established imprinted state.

It is becoming increasingly apparent that epigenetic modifications other than DNA methylation should also be considered for a part in plant genomic imprinting. Histone acetylation, deacetylation and methylation are all potential candidates. The possible role of Morpheus molecule 1 (MOM1) in plant imprinting is also of interest (Amendo et al., 2000). In *mom1* mutants a number of normally transcriptionally silent loci are reactivated, without any corresponding changes in DNA methylation. The transcriptional silencing activity of MOM1 could therefore act either downstream or independently of DNA methylation.

Much work remains to be done to uncover the mechanisms regulating genomic imprinting in plants. Conducting reciprocal crosses with DNA methyltransferase mutants and wild type plants and analysing the effect on seed development could elucidate the role DNA methyltransferases other than MET1 in the parent-of-origin effects. These experiments could also be extended to include *HDAC*, *HMTASE* and *mom1* mutants. It would also be informative to study if paternally inherited *MEA*, *FIE* or *FIS2* reporter transgenes are reactivated in these mutant backgrounds, or if the mutant pollen can rescue the *mea*, *fie* or *fis2* lethal seed phenotype. However, it remains a possibility that the primary regulation of imprinted gene expression is by as of yet unidentified factors. Furthermore, the study of genomic imprinting in *A.thaliana* is severely hindered by the small amount of known or suspected imprinted genes (*FIS* complex genes) and the lack of identified maternally imprinted loci. A screen for such genes could provide the key to genomic imprinting in plants.

8.3 The search for components of the genomic imprinting system in *A.thaliana*

The screen we designed and conducted in Chapter 7, to isolate mutations in components of the genomic imprinting system based on inheritable alterations in seed size, provided a large number of candidate lines in a short period of time. Many of these lines still need to be characterized. Excitingly, the one line which was studied in detail, 22.16, produced seed with an altered seed (paternal-excess) phenotype, which was expressed in a parent-of-origin dependent manner. Whether the paternal-excess phenotype is caused by a defect in the imprinting system still requires further investigation. However, the fact such an interesting mutant was identified highlights the great potential of this simple screen in identifying mutants of the sex-specific genomic imprinting system.

The work described in Chapter 5 also infers that factors such as plant age might affect genomic imprinting. Further study of the role of plant age might not only allow us a method by which to manipulate imprinting, it could also give us further insight into the mechanisms and evolution of imprinting in flowering plants.

8.4 Genomic imprinting in plants – the continuing story

Since the start of the work described in this Thesis there have been many exciting discoveries in the fields of genomic imprinting and epigenetics as a whole. However, there are still many pieces of the jigsaw missing and these must be uncovered to give the full picture of how and why genes are genomically imprinted in plants. This picture will give us an insight into the fascinating world of epigenetics and could provide us with tools to improve agriculture, including the introduction of apomixis and hybridization barriers into crop species.

Maternal plant age	Paternal plant age	Seed mass in µg
1	1	40
1	1	36
1	1	38
1	1	38
1	2	45
1	2	50
1	2	47
1	3	39
1	3	52
2	1	42
2	1	43
2	2	51
2	2	44
2	3	48
2	3	45
3	1	53
3	1	46
3	2	49
3	2	48
3	2	48
3	3	56
3	3	54

Appendix A.1
The mature mass of seed from [2x X 4x] crosses with plants of different ages. Data has a normal distribution (A^2=0.184, n=22, p=0.898) with the Anderson-Darling Normality Test.

Maternal plant age	Paternal plant age	Seed mass in µg
1	1	27
1	1	28
1	2	28
1	2	28
1	3	31
1	3	31
2	1	32
2	1	30
2	2	26
2	2	28
2	3	30
2	3	30
2	3	25
3	1	32
3	1	33
3	2	34
3	2	26
3	3	34
3	3	29

Appendix A.2
The mature mass of seed from [2x X hemi*MET1a/s*] crosses with plants of different ages. Data has a normal distribution (A^2=0.259, n=19, p=0.677) with the Anderson-Darling Normality Test.

Maternal plant age	Paternal plant age	Seed mass in µg
1	1	21
1	1	20
1	1	16
1	2	16
1	2	19
1	3	21
1	3	18
2	1	19
2	1	25
2	1	20
2	1	23
2	2	17
2	2	18
2	2	21
2	3	20
2	3	22
2	3	18
3	1	25
3	1	22
3	1	26
3	2	21
3	2	23
3	3	24
3	3	18

Appendix A.3

Mass of seed from [4x X 2x] crosses with plants of different ages. Data has a normal distribution (A^2=0..273, n=24, p=0.636) with the Anderson-Darling Normality Test.

Maternal plant age	Paternal plant age	Seed mass in µg
1	1	41
1	1	34
1	1	37
1	1	44
1	2	33
1	2	34
1	3	36
1	3	33
2	1	38
2	1	39
2	2	35
2	2	42
2	3	48
3	1	51
3	1	50
3	2	47
3	3	47
3	3	53
3	3	49

Appendix A.4

Mass of seed from [4x X 4x] crosses with plants of different ages Data has a normal distribution (A^2=0.556, n=19, p=0.130) with the Anderson-Darling Normality Test.

Maternal plant age	Paternal plant age	Seed mass in µg
1	1	16
1	2	14
1	2	13
1	3	15
1	3	14
1	3	13
2	1	17
2	2	17
2	2	16
2	2	15
2	3	16
2	3	14
3	1	21
3	1	19
3	2	15
3	2	19
3	3	16
3	3	18

Appendix A.5

Mass of seed from [4x X hemi*MET1a/s*] crosses with plants of different ages. Data has a normal distribution (A^2=0.634, n=19, p=0.079) with the Anderson-Darling Normality Test.

Maternal plant age	Paternal plant age	Seed mass in µg
1	1	36
1	1	37
1	2	39
1	2	29
1	2	37
1	3	32
1	3	34
2	1	34
2	1	39
2	2	39
2	2	33
2	2	34
2	3	37
3	1	42
3	1	42
3	2	54
3	3	48

Appendix A.6

Mass of seed from [hemi*MET1a/s* x 2x] crosses with plants of different ages. Data has a normal distribution (A^2=0.626, n=17, p=0.086) with the Anderson-Darling Normality Test.

Maternal plant age	Paternal plant age	Seed mass in µg
1	1	45
1	1	47
1	1	44
1	2	49
1	2	40
1	3	45
1	3	47
1	3	47
2	1	46
2	1	44
2	2	49
2	2	44
2	3	47
2	3	49
3	1	54
3	2	55
3	2	42
3	3	50
3	3	54
3	3	39

Appendix A.7

Mass of seed from [hemi*MET1a/s* X 4x] crosses with plants of different ages. Data has a normal distribution (A^2=0.339, n=20, p=0.463) with the Anderson-Darling Normality Test.

Maternal plant age	Paternal plant age	Seed mass in µg
1	1	28
1	1	32
1	2	28
1	2	32
1	3	28
1	3	29
1	3	26
2	1	29
2	1	30
2	2	28
2	2	30
2	3	31
2	3	27
3	1	36
3	1	36
3	2	30
3	3	37
3	3	32

Appendix A.8

Mass of seed from [hemi*MET1a/s* X hemi*MET1a/s*] crosses with plants of different ages. Data has a normal distribution (A^2=0.696, n=18, p=0.057) with the Anderson-Darling Normality Test.

Maternal plant age	Paternal plant age	Number of seed that germinated successfully[1]	Total number of seed[2]	Proportion of viable seed
1	1	30	31	0.967742
1	1	21	22	0.954545
1	1	18	20	0.9
1	2	14	15	0.933333
1	2	41	41	1
1	3	31	33	0.939394
1	3	26	27	0.962963
1	3	38	38	1
2	1	34	36	0.944444
2	1	32	32	1
2	2	41	41	1
2	2	39	39	1
2	3	30	30	1
3	1	23	25	0.92
3	1	27	30	0.9
3	1	23	23	1
3	2	17	23	0.73913
3	2	37	37	1
3	2	36	36	1
3	3	26	28	0.928571
3	3	11	11	1
3	3	4	8	0.5
3	3	42	42	1

[1] Number of seed in a pod that germinated successfully
[2] Total number of seed in a pod

Appendix B.1

The proportion of viable seed from [hemi*MET1a/s* X 4x] crosses with plants at different developmental stages.

Maternal plant age	Paternal plant age	Number of seed that germinated successfully	Total number of seed	Proportion of viable seed
1	1	1	8	0.125
1	1	2	20	0.1
1	1	35	42	0.833333
1	2	19	34	0.558824
1	2	0	35	0
1	3	0	28	0
1	3	23	41	0.560976
1	3	3	27	0.111111
1	3	3	28	0.107143
2	1	0	31	0
2	1	33	42	0.785714
2	2	26	36	0.722222
2	2	27	32	0.84375
2	2	22	33	0.666667
2	3	29	35	0.828571
2	3	29	37	0.783784
2	3	41	41	1
3	1	25	26	0.961538
3	1	21	26	0.807692
3	2	26	36	0.722222
3	2	9	13	0.692308
3	3	13	16	0.8125
3	3	36	37	0.972973

Appendix B.2
The proportion of viable seed from [4x X hemi*MET1a/s*] crosses with plants at different developmental stages.

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Parent-of-origin effects on seed development in *Arabidopsis thaliana* require DNA methylation

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SUMMARY

Some genes in mammals and flowering plants are subject to parental imprinting, a process by which differential epigenetic marks are imposed on male and female gametes so that one set of alleles is silenced on chromosomes contributed by the mother while another is silenced on paternal chromosomes. Therefore, each genome contributes a different set of active alleles to the offspring, which develop abnormally if the parental genome balance is disturbed. In *Arabidopsis*, seeds inheriting extra maternal genomes show distinctive phenotypes such as low weight and inhibition of mitosis in the endosperm, while extra paternal genomes result in reciprocal phenotypes such as high weight and endosperm overproliferation. DNA methylation is known to be an essential component of the parental imprinting mechanism in mammals, but there is less evidence for this in plants. For the present study, seed development was examined in crosses using a transgenic *Arabidopsis* line with reduced DNA methylation. Crosses between hypomethylated and wild-type diploid plants produced similar seed phenotypes to crosses between plants with normal methylation but different ploidies. This is consistent with a model in which hypomethylation of one parental genome prevents silencing of alleles that would normally be active only when inherited from the other parent – thus phenocopying the effects of extra genomes. These results suggest an important role for methylation in parent-of-origin effects, and by inference parental imprinting, in plants. The phenotype of biparentally hypomethylated seeds is less extreme than the reciprocal phenotypes of uniparentally hypomethylated seeds. The observation that development is less severely affected if gametes of both sexes (rather than just one) are 'neutralized' with respect to parent-of-origin effects supports the hypothesis that parental imprinting is not necessary to regulate development.

Key words: Seed, Endosperm, Methylation, Methyltransferase, *MET1*, Parental imprinting, *Arabidopsis thaliana*

INTRODUCTION

Parental imprinting plays an important role in the reproductive biology of mammals (Surani et al., 1990; Bartolomei and Tilghman, 1997) and flowering plants (Kermicle and Alleman, 1990; Haig and Westoby, 1991). For imprinted loci, the expression level of an allele depends on its parent of origin, due to differential epigenetic modifications imposed during male and female gametogenesis. Therefore male and female gametes are not equivalent, since each contributes a unique set of active alleles of imprinted genes to the offspring. As a consequence, in mammals, both a maternal and a paternal genome are required for development of a viable embryo, and a 1:1 ratio is necessary for normal development (Surani et al., 1990). The mechanisms by which imprinted loci are modified are still being uncovered but parent-specific DNA methylation has so far been associated with nearly all imprinted mammalian genes (reviewed by Neumann and Barlow, 1996; Jaenisch, 1997; Brannan and Bartolomei, 1999; Tilghman, 1999). Imprinting-associated methylation requires de novo modification of loci during gametogenesis, as well as post-fertilization propagation of imprints by maintenance methylation through many rounds of mitosis.

In flowering plants, the study of imprinting is complicated by the generation of two offspring – embryo and endosperm – in every seed, each with a different parental genome ratio. Endosperm plays an analogous role to the placenta in transferring maternal resources to the embryo (Brink and Cooper, 1947; Haig and Westoby, 1991; Lopes and Larkins, 1993; Berger, 1999), but unlike the placenta it is a separate fertilization product. Each pollen grain transmits two haploid sperm to the embryo sac, one of which fertilizes the egg to form a zygote with a ratio of 1 maternal to 1 paternal genome (1m:1p), while the other fertilizes a central cell containing two haploid polar nuclei (like the egg, derivatives of the female meiotic product) to form a primary endosperm cell with the constitution 2m:1p. There is strong though circumstantial evidence from many sources that imprinting directly effects endosperm development, with indirect consequences for embryo growth. Unlike the case in mammals, plant embryos can complete development and form viable adults with a constitution of 1m:0p or 2m:0p. In contrast, all sexually

reproducing angiosperms need maternal and paternal contributions to the endosperm, and even seeds producing parthenogenetic embryos sometimes require fertilization of the central cell (Sarkar and Coe, 1966; Nogler, 1984; Kermicle and Alleman, 1990). Furthermore, the 2m:1p ratio of the endosperm rather than the 1m:1p embryo ratio appears to be critical for normal seed development (Lin, 1984; Kermicle and Alleman, 1990; Haig and Westoby, 1991).

Most of the known imprinted genes in plants are expressed late in development of the persistent endosperm of maize; for example some members of the multi-gene family encoding zein storage proteins (Lund et al., 1995), and the *R* locus which regulates endosperm pigmentation (Kermicle, 1970). To date one gene from *Arabidopsis thaliana*, *MEDEA* (*MEA*) (Grossniklaus et al., 1998), has been shown to be imprinted (Kinoshita et al., 1999; Vielle-Calzada et al., 1999). Seeds inheriting a maternal *mea* mutation produce abnormal endosperm and embryo and abort, but the observation that mutant embryos can be rescued by culture (Vielle-Calzada et al., 1999) suggests to us that embryo lethality is a consequence of endosperm defects. Both studies of *MEA* imprinting agreed that expression in the endosperm is from maternal *MEA* alleles only, though results on embryo expression differed.

Many hypotheses have been advanced to explain the evolution of imprinting (reviewed by Hurst, 1997), but the most widely accepted is the parental conflict theory (Haig and Westoby, 1989, 1991; Moore and Haig, 1991). This interprets imprinting as a battle between maternal and paternal genomes over resource allocation from the mother to the embryo, proposed to arise because the reproductive fitness of a mother is greatest when she distributes resources equally among all her offspring, while a father benefits when maternal resources are concentrated in his own offspring. Therefore, the model predicts that maternally and paternally derived alleles will be selected to have opposite effects on embryo growth, with some growth promoters being paternally active and maternally silenced, and some growth inhibitors showing the opposite expression patterns. In mammals, most known imprinted genes (more than two dozen have been identified) are uniparentally expressed in the placenta, and many of these fit the parental conflict theory in having the predicted opposite effects on growth depending on parent of origin (Tilghman, 1999). In flowering plants, imprinted genes are predicted to directly affect the growth of endosperm – as this has primary responsibility for acquiring maternal resources for the seed – with mainly indirect consequences for the embryo (Haig and Westoby, 1989, 1991).

The *MEA* locus provides the only direct evidence so far concerning the role of imprinting in seed morphogenesis. However, there are likely to be more imprinted genes with a function in seed development, and evidence for these has been inferred from the effects of altering the balance of maternal and paternal genomes in the seed through crossing parents of different ploidies. In many species, an excess of paternal relative to maternal genomes appears to promote early growth of the endosperm, while maternal excess has the opposite effect (reviewed by Haig and Westoby, 1991). We found that in *Arabidopsis*, crosses between diploid (2x) and tetraploid (4x) plants in either direction produced viable seeds containing triploid embryos, and these had reciprocal phenotypes as predicted, with [4x × 2x] crosses (i.e. between a 4x seed parent and 2x pollen parent) producing small, underdeveloped endosperms and small embryos, and [2x × 4x] crosses generating large endosperms and embryos (Scott et al., 1998). Crosses between diploid and hexaploid plants resulted in similar but more extreme phenotypes, followed by abortion. Our results were consistent with a model in which maternal genomes contributed active alleles of endosperm growth inhibitors, and paternal genomes contributed active growth promoters, with the observed parent-of-origin effects on seed development reflecting dosage imbalances of these alleles. Although the crosses also altered the balance of parental genomes in the embryo, we concluded that the effects on embryo growth and viability were likely to be indirect, partly because of previous work, cited above, showing that the embryo is relatively insensitive to parental genome imbalance, and partly because the major morphological effects we observed were on endosperm development.

Little is known about the parental imprinting mechanism in plants, although there is evidence that as in mammals DNA methylation is involved. In maize endosperm, imprinted zein genes are only expressed when inherited from the seed parent, and these loci are methylated at fewer sites on maternally than paternally derived chromosomes (Lund et al., 1995). Differential methylation also corresponds with parent-specific expression of the *R* locus (Kermicle and Alleman, 1990; Finnegan et al., 1998). The methylation patterns of *MEA* have not been reported, but in seeds homozygous for a *decrease in DNA methylation 1* (*ddm1*) mutation, which reduces overall cytosine methylation by 70% (Vongs et al., 1993), a wild-type paternal *MEA* allele can rescue seeds carrying a normally lethal maternal *mea* mutation, implying that hypomethylation has activated the silenced paternal copy (Vielle-Calzada et al., 1999). However, *ddm1* mutations do not affect methyltransferase activity (Kakutani et al., 1995), and DDM1 has recently been found to be a member of the SWI2/SNF2 family of chromatin remodelling proteins (Jeddeloh et al., 1999). Furthermore, single-copy DNA sequences only lose methylation gradually in *ddm1* mutants through several generations of inbreeding (Kakutani et al., 1996). Therefore the *ddm1* mutation is likely to have its primary effect on chromatin configuration with only indirect effects on methylation. In addition, effects of the *ddm1* mutation alone on seed development have not been described beyond the observation that mutant seeds are viable.

In order to investigate the global role of methylation in parent-of-origin effects in seeds, we performed crosses using plants with DNA methylation reduced by a *METHYLTRANSFERASE 1* antisense (*MET1* a/s) transgene (MET1 is the predominant DNA methyltransferase in *Arabidopsis*) (Finnegan et al., 1996; Genger et al., 1999). If DNA methylation is essential to the imprinting mechanism in *Arabidopsis*, and if the antisense transgene prevents imprinting-specific methylation, we would expect hypomethylated plants to produce gametes in which imprinted alleles lose most or all of their silencing. For example, a hypomethylated pollen donor should provide sperm in which silencing is lifted from the alleles that are normally expressed from the maternal genome only, so the seed will contain extra active alleles of maternal-specific genes. Our results show that following reciprocal crosses between hypomethylated and wild-type plants, seed development is indeed affected as

predicted: crosses between hypomethylated 2x seed parents and wild-type (normally methylated) 2x pollen parents phenocopy the [2x × 4x] cross (Scott et al., 1998) in seed size and morphology, while crosses between wild-type 2x seed parents and hypomethylated 2x pollen parents phenocopy [4x × 2x] crosses. We conclude that methylation plays an important role in parent-of-origin effects, and by inference imprinting, in flowering plants. The reciprocal phenotypes also suggest that in each cross imprints are propagated in the genome derived from the wild-type parent. From this we infer that the antisense methyltransferase prevents establishment or maintenance of imprinting in gametes rather than propagation of imprinting after fertilization.

Jaenisch (1997) proposed that removal of imprints or of imprinted genes themselves should have few developmental consequences, as they exist in " 'paired sets' of genes involved in the same pathway" (e.g. of growth promoters and inhibitors, as predicted by Haig and colleagues). This has been difficult to test in mammals as embryos with reduced methylation die during gestation (Li et al., 1992). However, our results support a model in which imprinting is not essential to development, as we found that when both parents are hypomethylated seed phenotypes are less severe than the reciprocal phenotypes observed when only one is hypomethylated.

MATERIALS AND METHODS

Plant material

Plants were grown for 3-4 weeks at 22°C with a day length of 16 hours in a Fisons growth cabinet, then transferred to a glass house and grown at 24±2°C. Plants with wild-type methylation levels were C24 diploid (2x) A9-barnase (Paul et al., 1992), C24 tetraploid (4x), and Columbia hexaploid (6x) as described by Scott et al. (1998). Hypomethylated C24 plants were from the T3 generation of family 10.5, homozygous for the *Arabidopsis* methyltransferase I (*METI*) antisense construct under control of the cauliflower mosaic virus 35S promoter (Finnegan et al., 1996).

Cross pollinations

If the seed parent was male sterile (A9-barnase), open flowers were pollinated. If plants were male fertile, flower buds were emasculated 1 day prior to anthesis and pollinated 2 days later. Developing siliques were collected 2 to 8 days after pollination and processed as described below. Mature seeds were collected when pods were desiccated. Seeds were weighed using a Mettler UMT 2 microbalance (Mettler-Toledo, Leicester, UK).

Confocal laser scanning microscopy

Samples were prepared as in Braselton et al. (1996) and imaged at the University of Bath using an Axiovert 100M Zeiss LSM510 laser scanning microscope. Feulgen-stained samples were excited using an argon ion laser at 458 or 488 nm, and emissions detected at ≥515 nm. Images measuring 1024×1024 pixels were collected using a C-Apochromat 63×/1.2 water lens, saved in PSD format, and processed using Adobe Photoshop 4.0.1.

Southern analysis of genomic DNA

Genomic DNA was extracted from 0.1 g of leaf tissue using a Nucleon Phytopure Plant DNA Extraction kit (Nucleon, Biogenesis, Glasgow, UK) according to manufacturer's instructions. 100 ng of genomic DNA was digested overnight with *Msp*I or *Hpa*II and separated by electrophoresis on a 1% agarose gel. Southern analysis was performed as described by Southern (1975) with 0.4 M NaOH replacing 20× SSC as the buffer solution. The probe used contains a 180 bp repeat sequence from *Arabidopsis* centromeric DNA (Martinez-Zapater et al., 1986). Probe DNA was digested with *Pvu*II and a 500 bp fragment, consisting of the 180 bp repeat and 320 bp of pUC12 vector, was gel purified. The fragment was labelled by the random priming method using DIG11-UTP alkali label (Roche, Lewes, E. Sussex, UK) according to manufacturer's instructions. Hybridization was carried out at 65°C. Filters were washed at room temperature in 0.1× SSC and 0.1% SDS, and developed using an anti-digoxigenin antibody (Roche) and CPD Star substrate (Promega, Southampton, UK), according to manufacturer's instructions.

Detection of the *METI* antisense construct by PCR

Genomic DNA was extracted from 0.1 g of leaf tissue according to the small scale method of Edwards et al. (1991). DNA template (10 ng) was added to a 20 ml reaction mix containing 1.8 ml of 11× buffer (500 mM Tris-HCl pH 8.8, 120 mM NH_4SO_4, 50 mM $MgCl_2$, 75 mM β-mercaptoethanol, 0.05 mM EDTA, 11 mM dNTPs, 1.25 mg/ml DNAse-free BSA), 10 pmol of each primer (see below), and 1 U of Taq polymerase (Advanced Biotechnologies, Surrey, UK). The primers used were *METI*dF (5'-TAT AGG CCT GAG GAT GTT TCT GC-3') and *METI*bR (5'-AGG TCC ACC ATT GAT GAA GTC C-3'), which span an intron-containing sequence in the endogenous *METI* gene (Finnegan et al., 1993; accession no. L10692). Cycling conditions were 94°C for 3 minutes followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, carried out in an MJ Research PTC-200 Peltier Thermal Cycler. The reaction amplified a 1 kb product from the endogenous *METI* gene, while the antisense transgene generated an additional product of 0.7 kb.

RESULTS

To test the effect of decreasing methylation levels on seed development, we performed a series of crosses using transgenic plants homozygous for a *METHYLTRANSFERASE I* antisense construct (*METI* a/s) (Finnegan et al., 1996) as one or both parents. The transgenic line used was previously reported to have approximately 13% of the wild-type level of DNA methylation (Finnegan et al., 1996).

DNA methylation influences seed weight and germination

Crosses between two *METI* a/s plants, as well as reciprocal crosses between *METI* a/s and wild-type 2x parents, all produced viable seeds. Crosses using a *METI* a/s plant as one parent gave rise to hemizygous F_1 plants with reduced methylation levels (Fig. 1 and Finnegan et al., 1996). Dry weight and germination frequencies of seeds from the *METI* a/s crosses and wild-type interploidy crosses are compared in Fig. 2. As previously reported (Scott et al., 1998), seeds from [4x × 2x] and [2x × 4x] crosses are nearly always viable. [4x × 2x] seeds, which have double the normal dose of maternal relative to paternal genomes, are lighter than [2x × 2x] seeds (mean 15.3 μg compared with 20.8 μg), while [2x X 4x] seeds, with a double dose of paternal genomes, are heavier (53.7 μg; Fig. 2A). Seeds from [6x × 2x] and [2x × 6x] crosses, with more extreme maternal and paternal excess respectively, are shrivelled and inviable. Fig. 2B shows that crosses using *METI* a/s plants follow a similar trend. [2x × *METI* a/s] seeds – in which we predicted that normally maternal-specific alleles would be derepressed on the paternal chromosomes (thus phenocopying maternal excess) – are lighter than [*METI* a/s ×

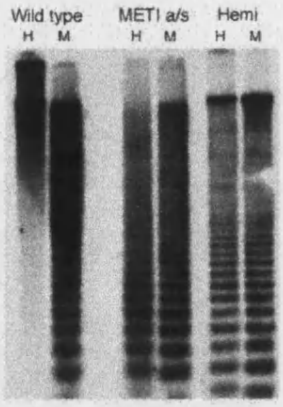

Fig. 1. Hypomethylation of genomic DNA associated with the *METI* a/s transgene. Southern analysis of DNA from wild-type (left), homozygous *METI* a/s (middle), and hemizygous *METI* a/s plants (right). DNA was digested with *Hpa*II (H) or *Msp*I (M); both cleave the sequence CCGG but the former is inhibited by cytosine methylation (McClelland et al., 1994). A 180 bp repeat from *Arabidopsis* centromeric DNA (Martinez-Zapater et al., 1986) was used as a probe.

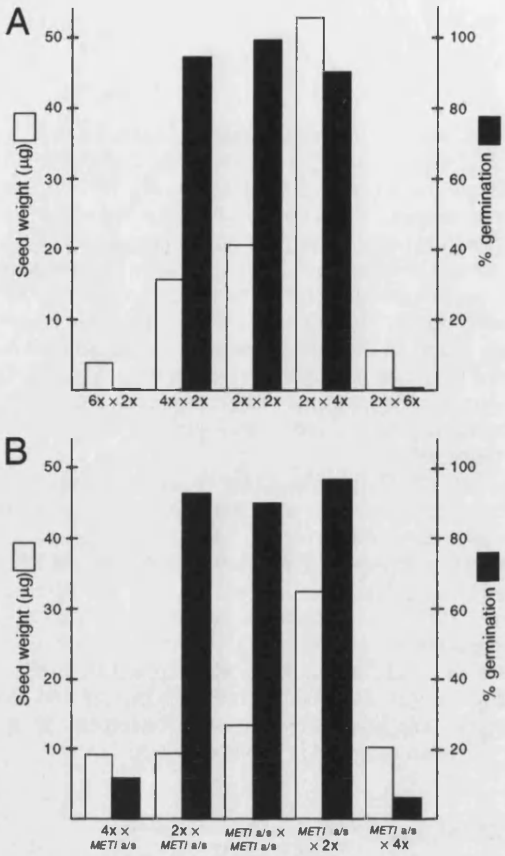

Fig. 2. Mean weights (white bars) and germination frequencies (black bars) of seeds from interploidy crosses (A) and crosses using *METI* a/s plants (B). (A) From left to right, mean weights are 4.0 μg (n=71), 15.3 μg (n=36), 20.8 μg (n=67), 53.7 μg (n=18), 5.7 μg (n=65); germination frequencies are 0, 95, 100, 90, 0% (n=20 for all). (B) From left to right, mean weights are 9.4 μg (n=28), 9.5 μg (n=19), 13.6 μg (n=22), 32.5 μg (n=46), 10.3 μg (n=27); germination frequencies are 12% (n=25), 93% (n=43), 90% (n=20), 97% (n=36), 6% (n=16).

METI a/s] seeds (9.5 μg compared with 13.6 μg), while [*METI* a/s × 2x] seeds, in which paternally expressed genes could be activated on the maternal chromosomes (phenocopying paternal excess), are heavier (32.5 μg). In all three crosses, most seeds are viable.

In the [4x × *METI* a/s] and [*METI* a/s × 4x] crosses, in which genomic imbalance is superimposed on hypomethylation, viability drops off sharply but is not reduced to 0 as for [2x × 6x] and [6x × 2x] crosses, and the mean seed weight is also higher than for these crosses.

Development of hypomethylated seeds

In normal *Arabidopsis* seeds, the endosperm proliferates as a syncytium until the embryo reaches heart stage, and then begins to cellularize from the micropylar pole (Mansfield and Briarty, 1990a,b; Scott et al., 1998; Brown et al., 1999; Berger, 1999). In our conditions cellularization begins at about 5 days after pollination (5 DAP). Several days before cellularization three regions of endosperm can be identified: central peripheral (composed of regularly spaced nuclei with associated cytoplasm lining the central region of the embryo sac); micropylar peripheral (nuclei embedded in a common cytoplasm surrounding the suspensor); and chalazal (a dense multinucleate tissue at the chalazal pole). The free-nuclear central peripheral endosperm often forms enlarged and sometimes multinucleate 'nodules' near the chalazal endosperm. An *Arabidopsis* seed at heart stage is shown schematically in Fig. 3.

Following interploidy crosses resulting in maternal excess, few endosperm nuclei are produced, peripheral endosperm cellularizes early (beginning at 4 DAP), chalazal endosperm is underdeveloped, no nodules are seen, and embryo differentiation is delayed (Fig. 4, [4x × 2x] and [6x × 2x]; Scott et al., 1998). In contrast, seeds with paternal excess produce peripheral endosperms with large numbers of nuclei which cellularize late (from 6 DAP) or never, and massively overgrown chalazal endosperm and nodules (Fig. 4, [2x × 4x] and [2x × 6x]; Scott et al., 1998).

Confocal microscopy shows that [*METI* a/s × *METI* a/s] seeds are not identical to wild type [2x × 2x]. [*METI* a/s × *METI* a/s] seeds show some features of seeds with maternal excess – for example small chalazal endosperm – but cellularization (at 5-6 DAP) is not early and proliferation of peripheral endosperm is not inhibited as in [4x × 2x] seeds (Fig. 4). Crosses in which a *METI* a/s plant is only one of the parents are more directly comparable with interploidy crosses. In [2x × *METI* a/s] seeds the endosperm underproliferates and cellularizes early (3-4 DAP), and these phenotypes are more extreme in [4x × *METI* a/s] crosses, with cellularization occurring at 2-3 DAP. In contrast, [*METI* a/s × 2x] seeds produce large peripheral endosperms with delayed cytokinesis (6-7 DAP), and overgrown chalazal endosperms and nodules, while [*METI* a/s × 4x] seeds have an even more pronounced paternal excess phenotype, with no cellularization at 10 DAP.

Endosperm development is quantified in Fig. 5, which shows numbers of peripheral endosperm nuclei counted after initiation of cellularization but before the embryo has begun to consume the endosperm. This stage occurs at a different number of days after pollination for each cross, ranging from 5 to 7 DAP, but is intended to reflect the maximum extent of endosperm proliferation. [*METI* a/s × *METI* a/s] seeds produce 598±126 (mean±s.e.m.) (n=3) peripheral endosperm nuclei,

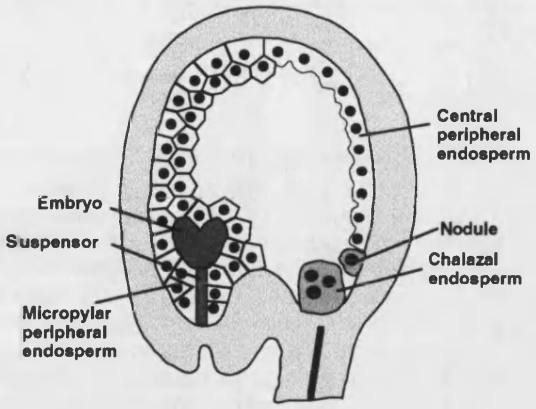

Fig. 3. Diagram of a seed containing a heart-stage embryo and cellularizing endosperm.

compared with 429±31 (n=3) for [2x × 2x] seeds. [2x × *METI* a/s] seeds produce less than half the number of peripheral endosperm nuclei observed in [*METI* a/s × *METI* a/s] seeds (227±17; n=6), while [*METI* a/s X 2x] seeds have more than twice the number (1,365±90; n=3). [4x × *METI* a/s] seeds have even fewer peripheral endosperm nuclei than [2x × *METI* a/s] – less than half the number again (97±10; n=4). On average, [*METI* a/s × 4x] seeds have slightly fewer peripheral endosperm nuclei than [*METI* a/s × 2x] (1,291±386; n=3), but in contrast to the latter, [*METI* a/s × 4x] seeds are mainly inviable. A similar trend is seen in [2x × 6x] compared with [2x × 4x] endosperms (Scott et al., 1998).

Crosses using *METI* a/s hemizygotes

For imprinting to affect gene expression in the developing seed, the different imprints on maternal and paternal chromosomes inherited by the primary endosperm nucleus must be propagated during endosperm proliferation. This raises the

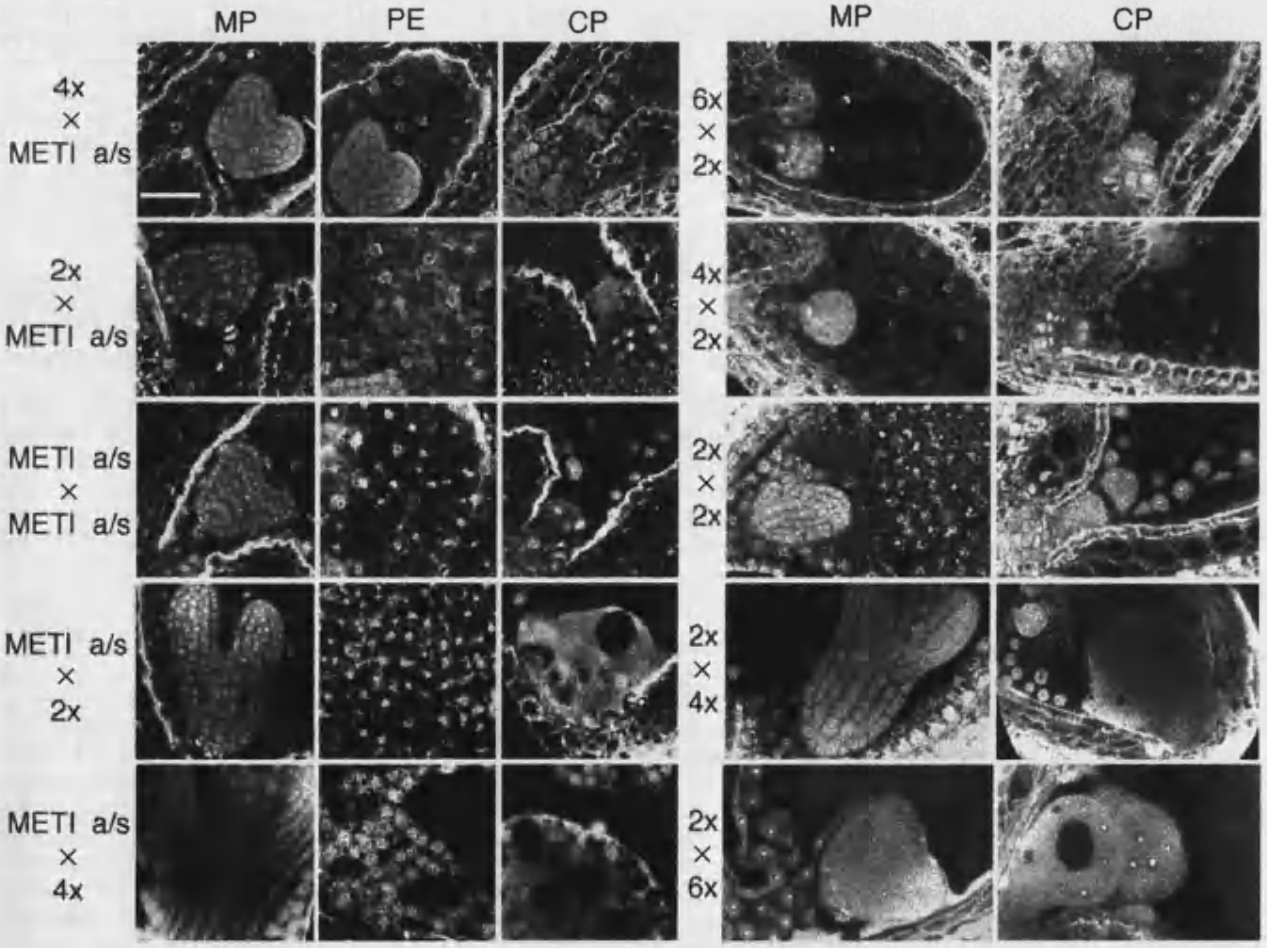

Fig. 4. Confocal micrographs of Feulgen-stained seeds from crosses with *METI* a/s plants (left) and interploidy crosses (right). Images were taken at different numbers of days after pollination (DAP) but reflect typical features of seeds with parental genome balance ([2x × 2x], 5 DAP), maternal genomic excess ([6x × 2x], 4 DAP and [4x × 2x], 5 DAP) or paternal excess ([2x × 4x], 6 DAP and [2x × 6x], 5 DAP). The insert in the lefthand image for [2x × 6x] shows free-nuclear peripheral endosperm. [2x × *METI* a/s] and [4x × *METI* a/s] seeds (both 5 DAP) have maternal excess phenotypes, while [*METI* a/s × 2x] and [*METI* a/s × 4x] seeds (both 7 DAP) have phenotypes typical of paternal excess (see text). [*METI* a/s × *METI* a/s] seeds (6 DAP) have features of both maternal and paternal excess. MP, micropylar pole; PE, central peripheral endosperm; CP, chalazal pole. Bar, 50 μm.

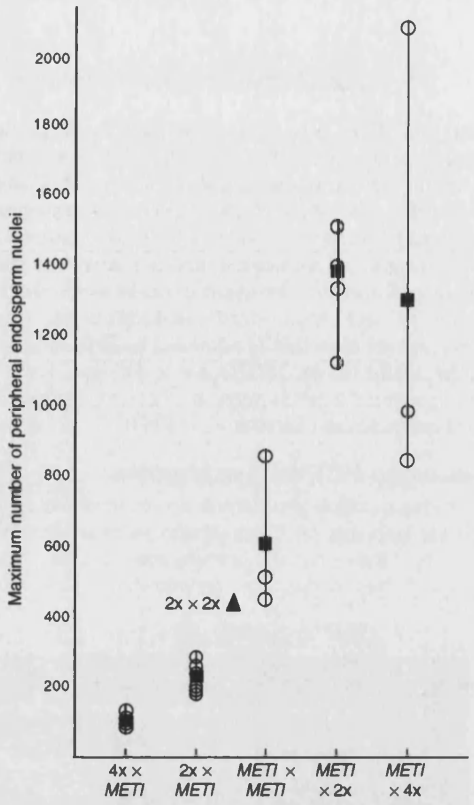

Fig. 5. Numbers of peripheral endosperm nuclei at maximum endosperm development in crosses with *METI* a/s plants. All data points are shown (open circles). The mean number of nuclei for each cross is also shown (solid squares). The mean number of endosperm nuclei in [2x × 2x] crosses (solid triangle) is shown for comparison.

possibility that a uniparentally transmitted *METI* a/s transgene could interfere with propagation of methylation imprints on all chromosomes in the endosperm, masking parent-specific effects. Therefore we tested whether presence of the transgene per se affected seed development, through reciprocal crosses between hemizygous *METI* a/s (hemi*METI* a/s) and wild-type 2x plants. (*METI* a/s plants that are hemizygous for the transgene remain hypomethylated, though to a lesser extent than homozygotes; Finnegan et al., 1996; and Fig. 1.) The results of these crosses are shown in Fig. 6. In 11 progeny of a [hemi*METI* a/s × 2x] cross, 6 inherited a copy of the transgene (Fig. 6A). Although approximately half of the seeds contained the transgene, all the seeds produced by the [hemi*METI* a/s × 2x] cross were similar in size, morphology, and development (Fig. 6B,C). Likewise, we saw no differences among seeds produced by the [2x × hemi*METI* a/s] cross. However, there were differences between the crosses. [2x × hemi*METI* a/s] crosses produced small seeds (mean weight 10.9 μg; n=26) with phenotypes similar to those of [2x × (homozygous) *METI* a/s] seeds, while [hemi*METI* a/s × 2x] seeds were large (28.6 μg; n=39) and resembled [*METI* a/s × 2x] seeds (Fig. 6B-D; cf. Figs 2, 4, 5). Endosperm proliferation in crosses using one hemi*METI* a/s parent followed the same trends as crosses in which one parent was homozygous for the transgene. [hemi*METI* a/s × 2x] seeds produced 810±23 (mean±s.e.m.) peripheral endosperm nuclei (n=6), higher than the mean for [*METI* a/s × *METI* a/s] crosses, while [2x × hemi*METI* a/s] seeds generated 242±28 peripheral endosperm nuclei (n=6), lower than the [*METI* a/s × *METI* a/s] mean (Figs 5 and 6C).

DISCUSSION

Relationship between methylation and imprinting

In crosses using a *METI* a/s plant as only one parent, seed weights, germination frequencies, and developmental patterns including endosperm proliferation and timing of cellularization all show that hypomethylation closely phenocopies the effects of interploidy crosses (Figs 2, 4, 5). [*METI* a/s × 2x] seeds have a strong paternal excess phenotype with high seed weight, many endosperm nuclei, delayed endosperm cellularization, and overgrown chalazal endosperm, although both parents are diploid, and the seed is nourished by a hypomethylated mother which suffers a variety of defects in vegetative and floral development (Finnegan et al., 1996). This behaviour is consistent with a model in which hypomethylation of the maternal genome in *METI* a/s plants has prevented silencing of endosperm-promoting genes which would normally only be expressed from the paternal genome (Haig and Westoby, 1989, 1991; Scott et al., 1998). Meanwhile, the wild-type paternal genome contributes its normal complement of silenced endosperm-inhibiting genes and active endosperm-promoting genes. The net effect according to the model is that the endosperm has an excess of imprinted alleles that behave as if they were inherited from the father, thus phenocopying an excess of paternal genomes.

The reciprocal phenotypes of [4x × 2x] and [2x × 4x] crosses can only be explained if female and male gametes contribute different sets of active alleles or else different complements of gene products (i.e. cytoplasmic factors) to the seed. (In the first case the alleles are not necessarily expressed in the gametophyte, but need to be transmitted to the seed with a potential for expression – although some may fall into both categories, like *MEA*, which is transcribed in the female gametophyte as well as being transmitted to the seed with maternal alleles competent for expression; Grossniklaus et al., 1998.) Similarly, the reciprocal phenotypes of [2x × *METI* a/s] and [*METI* a/s × 2x] crosses can only be explained if uniparental hypomethylation affects sex-specific gene expression in a way that closely phenocopies interploidy crosses. Formally it is possible that the interploidy cross phenomena are due to a dosage imbalance of genes expressed exclusively in the central cell and sperm whose products are carried over to the endosperm. It is then possible that hypomethylation allows ectopic expression of these gametophytic genes in the wrong sex, so that sperm-specific genes are activated in the central cell and vice versa, and that deregulated gametophytic expression alone is responsible for the phenocopy of interploidy crosses. These scenarios would be consistent with the reciprocal phenotypes without involving imprinting. However, we consider this an unlikely explanation for all of our findings, particularly those resulting in paternal excess and its phenocopy. Plant sperm and generative cells (sperm precursors) are characterized by condensed chromatin,

little cytoplasm, and few organelles, and very few generative cell- or sperm-specific proteins have been identified (McCormick, 1993; Blomstedt et al., 1996). It is difficult to conceive how the paternal excess phenotype seen following interploidy crosses could be explained solely by an overdose of gene products specific to sperm, or how ectopic production of sperm-specific gene products in the embryo sac could have the observed effects on seed development. Furthermore, gametes produced by *METI* a/s plants are fertile, which one might not expect if there was general deregulation of sex-specific genes. Consequently, we consider disruption to the balance of expression of imprinted genes to be the most likely explanation for the results of interploidy crosses and uniparental hypomethylation.

Based on the above hypothesis that a hypomethylated genome has a similar effect to adding a genome of the opposite sex, one can predict that crossing a hypomethylated plant with a polyploid plant should have even more severe consequences for seed development. To test this we performed reciprocal crosses between hypomethylated diploid plants and normally methylated tetraploid plants. Seeds produced by [*METI* a/s × 4x] crosses usually abort and all have a strong paternal excess phenotype, resembling offspring of normally methylated [2x × 6x] crosses. In the [*METI* a/s × 4x] cross, a hypomethylated maternal genome, which phenocopies excess paternal genomes, is added to a real excess of paternal genomes, apparently pushing this cross towards more extreme paternal excess. Similarly, seeds from [4x × *METI* a/s] crosses have a stronger maternal excess phenotype than [2x × *METI* a/s] seeds.

So far the few studies of the role of methylation in parent-of-origin effects on seed development have focussed on single imprinted loci, e.g. zein genes (Lund et al., 1995), the *R* locus (Kermicle, 1970; Kermicle and Alleman, 1990; Finnegan et al., 1998), and *MEA* (Vielle-Calzada et al., 1999). The first two genes are not involved in seed morphogenesis, and the latter study is complicated by likely effects of *ddm1* on chromatin configuration as well as methylation (Jeddeloh et al., 1999). To our knowledge the work here presents the first evidence that methylation has a general role in parent-of-origin effects in plants, most likely reflecting its role in regulating expression of many imprinted genes on both maternal and paternal chromosomes.

Imprints can be propagated in seeds with one hypomethylated parent

For some mammalian imprinted genes, parent-specific methylation has been traced from the sperm and eggs of parents to the somatic tissues of offspring, indicating that the methylation patterns inherited from each parent are maintained after fertilization (Jaenisch, 1997; Tilghman, 1999). Therefore we envisaged the *METI* a/s transgene as having several possible consequences for parent-of-origin effects: it could prevent establishment or maintenance of methylation during gametogenesis, or it could prevent maintenance of parent-specific methylation in the seed (or both). If presence of a transgene in the seed abolished propagation of imprints, then we would expect reciprocal crosses between wild-type and hypomethylated plants to have the same phenotypes. This is not the case, and the complementary phenotypes suggest that imprints have been maintained on the chromosomes inherited from the wild-type parent (though this does not mean that endogenous *METI* has no role in propagating imprints post-fertilization, since it is possible that the 35S promoter driving the antisense construct is not active early in seed development).

Further evidence that the transgene per se does not interfere with imprinting maintenance in the seed comes from reciprocal crosses between wild-type diploid plants and *METI* a/s plants hemizygous for the transgene (hemi*METI* a/s). These produced similar (though weaker) phenotypes to crosses between wild-type and homozygous *METI* a/s parents, with [2x × hemi*METI*

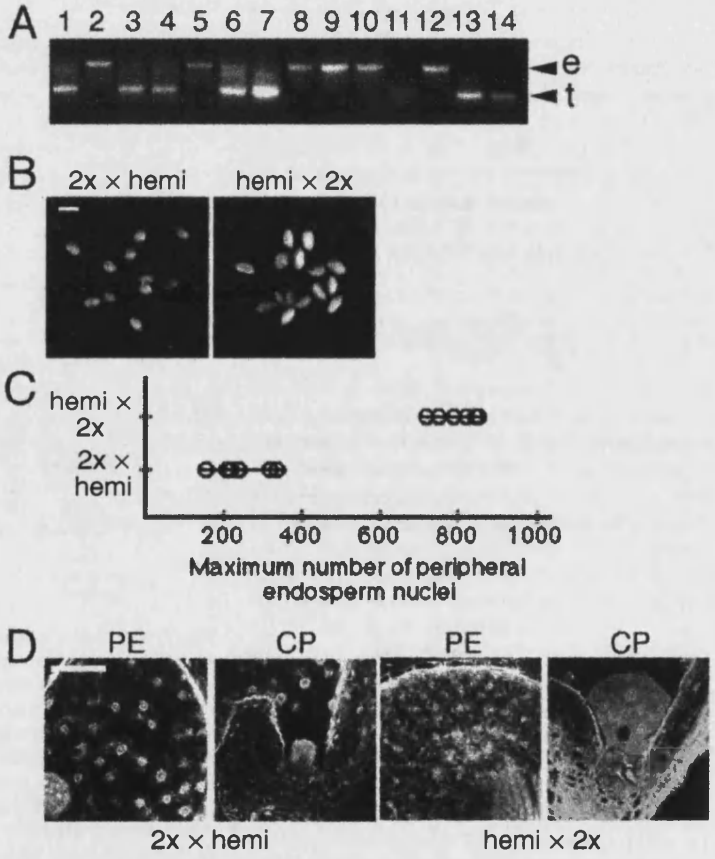

Fig. 6. Reciprocal crosses between wild-type 2x and hemizygous *METI* a/s plants. (A) PCR products showing segregation of the *METI* a/s transgene in progeny of a [hemi*METI* a/s × 2x] cross (lanes 1-11); lane 12, wild type; lane 13, *METI* a/s homozygote; lane 14, *METI* a/s hemizygote. In our conditions the transgene was preferentially amplified over the endogenous gene. e, endogenous *METI* gene; t, transgene. (B) Mature seeds from [2x × hemi*METI* a/s] and [hemi*METI* a/s × 2x] crosses, showing the single class of seed size within each cross. Bar, 1 mm. (C) Number of peripheral endosperm nuclei (all data points shown as open circles; cf. Fig. 5). (D) Confocal micrographs of Feulgen-stained seeds. [2x × hemi*METI* a/s] seeds have a maternal excess phenotype, while [hemi*METI* a/s × 2x] seeds have a paternal excess phenotype; cf. Fig. 4. PE, peripheral endosperm; CP, chalazal pole. Bar, 50 μm.

a/s] phenocopying [4x × 2x] crosses and [hemi*METI* a/s × 2x] resembling [2x × 4x] (Fig. 6B-D). Strikingly, within each cross with a hemi*METI* a/s parent, there was a single class of seed as measured by size and weight, morphology, and number of peripheral endosperm nuclei, although only half the progeny inherited the transgene (Fig. 6A). Therefore, the seed phenotypes were consistent with imprints being maintained, whether the seed contained a *METI* a/s construct or had a wild-type genotype. The *Arabidopsis* genome has been found to regain methylation slowly after a *METI* a/s transgene or *ddm1* mutation is segregated away (Vongs et al., 1993; Finnegan et al., 1996), which has been interpreted as reflecting a slow rate of de novo methylation. It could be argued that our results reflect lack of remethylation in seeds only one generation after losing the transgene. However, de novo methylation would not be required to propagate imprints on methylated DNA inherited from a wild-type parent, and the reciprocal phenotypes of [2x × hemi*METI* a/s] and [hemi*METI* a/s × 2x] crosses again imply that imprints are maintained on chromosomes transmitted by these plants. Therefore we conclude the hemi*METI* a/s results show that the transgene per se does not affect maintenance of methylation imprints in the seed. Taken together our results show that the *METI* a/s transgene prevents establishment or propagation during gametogenesis of methylation associated with parent-of-origin effects. This is in contrast to the results described by Vielle-Calzada et al. (1999), who concluded that the *ddm1* mutation abolished maintenance of *MEA* imprinting in the seed rather than establishment of the imprint in pollen.

Timing of methylation associated with parent-of-origin effects

Our results are consistent with a role for METI in establishing imprinting-associated methylation, but it is not known when and where during reproductive development this enzyme is active. In mouse, investigation of the major methyltransferase *Dnmt1*, among other evidence, indicated that imprints are most likely imposed during meiosis (Mertineit et al., 1998; Brannan and Bartolomei, 1999). Nothing is known of when imprinting might be set in plants, but as it must occur when male and female gametes or their precursors are separated, it could be any time between floral organ differentiation and fertilization. It is notable that following crosses between hemi*METI* a/s and wild-type plants, each seed develops according to the methylation status of its parents regardless of whether it inherits a transgene; one explanation is that at least some element of parent-specific methylation may be set before the nuclear divisions of meiosis. Another possibility is that gametes do not normally express *METI* but inherit METI protein from the diploid spore mother cells: this could also explain why the genotype of the parent plant rather than the meiotic product is reflected in the seed phenotype. Analysis is further complicated by lack of information about endogenous *METI* expression as well as the timing and location of *METI* a/s activity: e.g. the 35S promoter driving the antisense construct is probably not active during pollen development (Wilkinson et al., 1997). METI mRNA and protein localization in wild-type and transgenic plants would help distinguish between the alternatives.

Development of biparentally hypomethylated seeds

Following [*METI* a/s × *METI* a/s] crosses 90% of seeds are viable, although the parent plants are hypomethylated by 85% (Finnegan et al., 1996). Therefore it is possible in plants to test the effects of relaxing methylation-dependent imprinting – an experiment impossible in animals, as mice with methylation

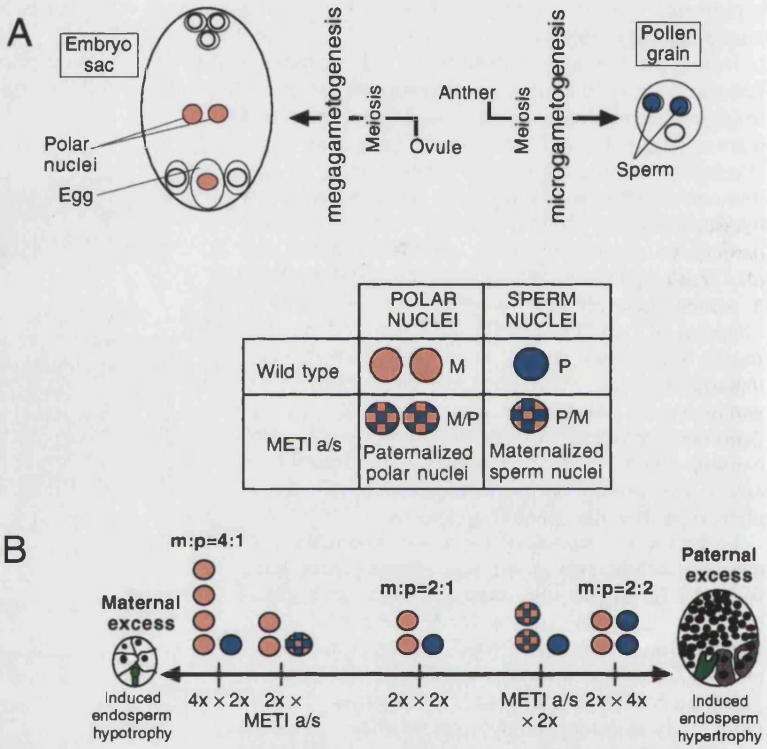

Fig. 7. Model of the effect of global DNA hypomethylation on parental imprinting in *Arabidopsis*. (A) Normally endosperm contains a ratio of two maternal genomes, contributed by the polar nuclei, to one paternal genome, contributed by the sperm. In maternal genomes, maternal-specific imprinted genes are active, while paternal-specific genes are repressed. Imprinted genes contributed by the paternal genome have a complementary expression pattern. When maternal genomes are contributed by a *METI* a/s parent, the paternal-specific genes are expected to be largely derepressed, producing a 'paternalized' genome. Similarly a *METI* a/s pollen parent is expected to contribute a 'maternalized' genome. (B) Interploidy crosses (e.g. [4x × 2x] or [2x × 4x]) result in seeds with extra maternal or paternal genomes, and therefore extra doses of active maternal or paternal alleles of imprinted loci. Maternal or paternal excess has dramatic and complementary effects on seed development, resulting in small seeds with small endosperms and large seeds with overgrown endosperms, respectively (described by Scott et al., 1998). A diploid *METI* a/s parent does not contribute extra genomes but appears to contribute extra doses of active maternal- or paternal-specific genes, resulting in phenotypes similar to those produced by parental genomic imbalance.

reduced by 70% through targeted mutation of *Dnmt1* die early in embryogenesis (Li et al., 1992). We found that seeds from [*METI* a/s × *METI* a/s] crosses were abnormal, but in seed weight, extent of endosperm proliferation, and timing of endosperm cellularization more closely resembled [2x × 2x] seeds than those produced by crosses between one *METI* a/s and one wild-type 2x plant (Figs 2, 4, 5). This observation, along with the complementary phenotypes of crosses in which only one parent is hypomethylated, suggests that the *METI* a/s transgene deregulates the sets of antagonistic growth control genes predicted to be subject to parent-specific expression by the parental conflict theory (Haig and Westoby, 1989, 1991; Moore and Haig, 1991). Jaenisch (1997) proposed that according to the theory, "removal of all imprints should have no ill effect", and "Imprinting has no intrinsic role in mammalian development...Imprinted genes are viewed as 'paired sets' of genes involved in the same pathway where removal of the set has little or no developmental consequences." Our data is consistent with the hypothesis that removal of imprinting in parents indeed has little effect on development (compared with removal of imprinting in just one parent). We have no evidence concerning removal of the sets of genes per se; instead, we infer that biparental hypomethylation in effect adds sets of antagonistic genes.

Although [*METI* a/s × *METI* a/s] crosses produce seeds that appear more normal than [2x × *METI* a/s] or [*METI* a/s × 2x] crosses, they contain small chalazal endosperms (Fig. 4) and weigh less than wild-type seeds (Fig. 2), both features of seeds from interploidy crosses which have inherited extra maternal genomes (Scott et al., 1998). This is an unexpected result, as one might predict a paternal rather than maternal excess phenotype for the following reason. If imprinting-specific methylation is lost equally on the maternal and paternal genomes in *METI* a/s plants, then the effective genome ratio in a [*METI* a/s × *METI* a/s] endosperm should be 3m (2m from the central cell and 1m equivalent from the hypomethylated sperm):3p (1p from the sperm and 2p equivalent from the hypomethylated central cell), making it equivalent to the 2m:2p ratio found in a [2x × 4x] cross. One aspect of the phenotype, however, is consistent with this prediction: the number of peripheral endosperm nuclei in [*METI* a/s X *METI* a/s] crosses is higher than in [2x × 2x] and about six-fold greater than in [4x × 2x] crosses (Fig. 5 and Scott et al., 1998).

We do not know why [*METI* a/s × *METI* a/s] seeds appear to show a combination of maternal and paternal excess phenotypes, but there are several factors which may contribute to this. Demethylation is not complete in *METI* a/s plants (Finnegan et al., 1996), perhaps in part because of other methyltransferases in the *Arabidopsis* genome which are not affected by the *METI* a/s transgene (Genger et al., 1999). Partial demethylation could affect gamete genomes or individual sequences unequally. In addition, some genes may even become hypermethylated in a *METI* a/s background, like the (non-imprinted) *SUPERMAN* locus (Jacobsen and Meyerowitz, 1997). Finally, due to the complicated regulation of imprinted genes, global DNA hypomethylation in mouse can repress as well as activate imprinted alleles (Li et al., 1993; Tilghman, 1999). It is conceivable the same occurs in plants, although evidence presented above suggests that the overwhelming effect of hypomethylation is to activate normally silent imprinted alleles.

A model for action of DNA methylation in parental imprinting in plants

The results above show that DNA methylation is an important part of the parent-of-origin effect in *Arabidopsis* following interploidy crosses, consistent with an essential role for methylation in the parental imprinting mechanism in flowering plants. Global DNA hypomethylation appears to derepress genes contributed to the seed by the polar nuclei that would normally be active only in the male genome, and derepress genes contributed by the sperm that would normally be female-specific. This has an effect of 'paternalizing' the female genome and 'maternalizing' the male genome (Fig. 7A). The phenotypic consequences are shown in Fig. 7B. We conclude that it is possible through uniparental hypomethylation to modify seed development and ultimately, size, most likely through lifting the silencing on parentally imprinted genes.

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